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Karolinska Institutet, Stockholm, Sweden

Exercise-induced molecular mechanisms in untrained and life-long highly trained individuals

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Karolinska Institutet

Stockholm 2020

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Published by Karolinska Institutet.
Printed by US-AB, 2020
Cover Art by Amagoia Agirre – www.amagoiaagirre.com

ISBN 978-91-7831-865-0

Exercise-induced molecular mechanisms in untrained and life-long highly trained individuals

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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ABSTRACT

Regular physical activity results in extensive systemic and functional adaptation effects in the human body that contribute to physical performance, such as muscular strength and endurance, and can have beneficial health effects on the cardiorespiratory, vascular and immune systems as well as on bone density and metabolic control. Adaptation to regular exercise requires translation of exercise-related signals into molecular responses, including epigenetic modification and other molecular processes. Over time, such processes result in accumulating cellular and sub-cellular biochemical and structural changes of tissues and organ systems. Exercise-related signals that challenge the body as a functional system include hypoxia, flux of energy rich substrates, changes in body temperature, lactate-induced pH changes, changed abundance of metabolites and mechanical shear stress. To overcome such challenges and improve future preparedness, tissues adapt for example by increased mitochondrial content in skeletal muscle, optimized temperature management and circulation, increased plasma volume and altered cell content in circulating blood, increased vascularization or by structural reinforcement and increased ability to develop force in skeletal muscle. In life-long trained athletes, the adaptations can result in outstanding, sports-specific performance. However, not all contributing mechanisms and sports-specific differences are well understood, especially in the context of elite athletes.

The results presented in this thesis are based on five papers in which skeletal muscle biopsies (**paper I-V**) and blood samples (**paper V**) were collected at different timepoints around acute (**papers I, IV, V**) and long-term exercise (**papers I, II, III**). The subjects in **papers I-II** were young, healthy, normally active men and women, in **papers III-V**, the subjects were healthy middle-aged men and women, either with a life-long history of sedentary lifestyle or high-level physical activity in endurance- or resistance- based sports. Five experimental models were used: acute bipedal cycling for 60 minutes (**paper I**), a 12-week unilateral leg extension endurance training protocol consisting of 4x45min of exercise per week (**paper I**), a 10-week protocol with unilateral leg press and leg extension resistance training at 70-85% 1RM (**paper II**), acute bipedal cycling for 30 minutes and acute leg extension at 80% 1RM in a cross-over design (**papers IV-V**). **Paper III** consisted of a cross-sectional study design without any acute intervention. Collected samples were analyzed by qPCR, western blot (**papers I-II**), bisulfite-transformation, pyrosequencing and phosphorylation analysis (**paper II**), immunohistochemistry, citrate synthase assay, RNA sequencing (**papers III-V**) and FACS sorting (**paper V**).

The overall aim of this thesis was to investigate molecular mechanisms that support and maintain life-long high-level adaptations to exercise training. In **paper I**, the translation of the biomechanical impulse from contracting skeletal muscle into downstream molecular signaling was investigated. In brief, it was shown that the previously described STARS signaling pathway, which links biomechanical and molecular effects is upregulated immediately following acute cycling exercise and that long-term training neither blunts nor amplifies such an acute response pattern. Furthermore, for the first time it was shown that there is no difference between men and women in STARS response. In **paper**

II we investigated how these adaptations can be "memorized" after a period of detraining. We found increased levels of phosphorylation of key genes in previously trained muscle and identified differences in gene expression of PGC-1α and other genes important for myogenesis, suggesting potential mechanisms for a "muscle memory". In a cross-sectional investigation in **paper III**, using global transcriptome analysis, gene ontology and genome-scale metabolic modelling we show that life-long high-level adaptation to endurance exercise is very different from the adaptation following life-long high-level resistance training, particularly in pathways related to the prevention of metabolic diseases, such as type 2 diabetes, and that differences between resistance training and sedentary behavior is comparably small. Furthermore, we found significant sex differences between untrained men and women and that these differences were markedly smaller comparing long-term trained men and women. We also showed that metabolically impaired individuals who submit to short-term endurance training become more similar to long-term endurance trained subjects and identified potential exercise-responsive genes. In **paper IV** we identified acute exercise-specific patterns of differential gene expression and identified important transcription factor motifs that contribute to these differences in long-term trained athletes. We showed that acute resistance exercise results in generally larger numbers of differentially expressed genes compared to acute endurance exercise and identified amongst others HIF1A and MYFfamily motifs as highly relevant to endurance and resistance exercise respectively. Furthermore, we identified groups of candidate genes that are especially relevant to these transcription factors and show that these genes are functionally closely connected. We also demonstrate that endurance trained athletes handle the metabolic stress of energy production differently than strength trained athletes and untrained subjects, surprisingly by a large-scale downregulation of metabolites and enzymes engaged in energy production processes immediately following acute endurance exercise, confirming the uniqueness of endurance athletes proposed in **paper III**. In **paper V** we investigated how high-level long-term training modulates the response of circulating immune cells to acute endurance and resistance exercise. We show that life-long high-level endurance athletes increase their numbers of circulating monocytes to a significantly larger extent and that the recovery of numbers of macrophages is significantly lower compared to untrained controls. Additionally, we show significant differences between the immune system response to acute endurance or resistance exercise. Furthermore, we cross-referenced immune cell concentrations in circulating plasma with the expression of immune cell marker genes in skeletal muscle and cytokine signaling in blood and demonstrated a higher enrichment of immune cell mobility related functional groups of genes in untrained control subjects compared to long-term trained athletes and a generally higher coordination of these functional groups of genes in response to acute endurance exercise compared to acute resistance exercise across all groups.

In conclusion we show that life-long trained endurance athletes handle metabolic challenges in a unique way and have a resting transcriptome largely different to strength trained and control individuals. Furthermore, we suggest the phosphorylation of proteins related to protein synthesis as potential molecular mechanism for a muscle memory effect. Finally, we show, that long-term training does not blunt STARS pathway-based signal translation of mechanical contraction.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers which are referred to in the text by their roman numerals:

I. **Reitzner SM**, Norrbom J, Sundberg CJ, Gidlund E-K. Expression of striated activator of rho-signaling in human skeletal muscle following acute exercise and long-term training. *Physiol Rep*. 2018;6(5):e13624. doi:10.14814/phy2.13624.

II. Moberg M, Lindholm ME, **Reitzner SM**, Ekblom B, Sundberg CJ, Psilander N. Exercise Induces Different Molecular Responses in Trained and Untrained Human Muscle. *Med Sci Sports Exerc*. 2020, Aug; 52(8). doi: 10.1249/mss.0000000000002310.

III. Chapman MA, Arif M, Emanuelsson EB, **Reitzner SM**, Lindholm ME, Mardinoglu A, Sundberg CJ. Skeletal Muscle Transcriptomic Comparison between Long-Term Trained and Untrained Men and Women. *Cell Rep*. 2020;31. doi:10.1016/j.celrep.2020.107808

IV. **Reitzner SM**, Emanuelsson EB, Kaczkowski B, Arif M, Kwon ATJ, Mardinoglu A, Arner E, Chapman MA, Sundberg CJ. A systems biology approach to the effect of acute endurance and resistance exercise in skeletal muscle of life-long trained athletes. *Manuscript.*

V. **Reitzner SM**, Ruiz de Assin E, Emanuelsson EB, Barbieri L, Rundqvist H, Sundberg CJ. Life-long training modulates the immune system response to acute endurance and resistance exercise. *Manuscript.*

Publications by the author not included in this thesis:

Reitzner SM, Hengevoss J, Isenmann E, Diel P. Modulation of exercise training related adaptation of body composition and regulatory pathways by anabolic steroids. *J Steroid Biochem Mol Biol*. 190 (2019) 44–53. doi:10.1016/j.jsbmb.2019.03.023.

Rundqvist H, Veliça P, Barbieri L, Gameiro PA, Bargiela D, Gojkovic M, Mijwel S, **Reitzner SM**, Wulliman D, Ahlstedt E, Ule J, Östman A, Johnson RS. Cytotoxic T-cells mediate an exercise-induced reduction in tumor growth. *eLife*. Under review.

CONTENTS

LIST OF ABBREVIATIONS AND DEFINITIONS

1RM	One Repetition Maximum
ANOVA	Analysis of Variance
Art.	*(lat.)* articulatio
ATP	Adenosine Triphosphate
CD	Cluster of Differentiation
CNS	Central Nervous System
CpG site	Region of DNA where Cytosine is followed by Guanine
CPT	Cell Preparation Tube
CREB1	CAMP Responsive Element Binding Protein 1
CS	Citrate Synthase
CSA	Cross-sectional Area
DEG	Differentially Expressed Gene
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
EE/END	Acute Endurance Exercise
ERRα	Estrogen Related Receptor Alpha
FACS	Fluorescence-activated Cell Sorting
FANTOM	Functional Annotation of the Mammalian Genome
FBS	Fetal Bovine Serum
FC	Female Control Group
FE	Female Endurance group
GEM	Genome-scale Metabolic Modelling
GO	Gene Ontology
GSEA	Gene Set Enrichment Analysis
HIF1A	Hypoxia-inducible Factor 1A
IPC	Ischemic Preconditioning
log(FC)	Logarithm of Fold Change
M.	*(lat.)* musculus
MARA	Motif Activity Response Analysis
MC	Male Control Group
ME	Male Endurance Group
MetS	Metabolic Syndrome
MHC	Myosin Heavy Chain

MRI	Magnetic Resonance Imaging
mRNA	Messenger RNA
MRTF-A	Myocardin-related Transcription Factor A
MS	Male Strength Group
MYF	Myogenic Factor
NGT	Normal Glucose Tolerance
PBMC	Peripheral Blood Mononucleate Cell
PBS	Phosphate Buffered Saline
PGC-1α/PPARGC1A	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-Alpha
PIANO	Platform for Integrative Analysis of Omics Data
qPCR	Quantitative Polymerase Chain Reaction
RE/RES	Acute Resistance Exercise
Regulome	The sum of all regulatory components of a cell
Rep	A repetition; in exercise science consists of a concentric and an eccentric movement (moving a weight against a resistance and a return to staring position).
REVIGO	Reduce and Visualize Gene Ontology
RNAseq	Ribonucleic Acid Sequencing
Set	A certain number of repetitions performed in sequence
SRF	Serum Response Factor
STARS	Striated Activator of Rho-Signaling
T2D	Type 2 Diabetes
TCA cycle	Tricarboxylic Acid Cycle
TCSBN	Tissue and Cancer Specific Biological Networks
TF	Transcription Factor
V.	*(lat.)* vena
VO_2-peak	Peak Oxygen Uptake

1 BACKGROUND

Elite athlete level physical performance requires consistent, systematic exercise training over a long period of time. From the beginning of an individual training career to peak of performance a number of important molecular processes contribute to adaptations which can result in maintenance and improvement of performance capabilities. In addition to performance, physical activity has numerous health benefits in the cardiorespiratory, vascular and immune systems as well as improved metabolic control which has positive influence on preventing diseases such as cardiovascular, type 2 diabetes and metabolic syndrome[1–6]. Adaptation to regular exercise involves translation of exercise-related biochemical and biophysical stimuli into molecular responses that are to a great extent regulated at the level of gene expression and protein abundance partly involving epigenetic modifications and subsequently affecting more long-lasting structural changes in tissues and organ systems[7]. Exercise-related stimuli include hypoxia, flux of energy-rich substrates, elevation of body temperature, increased concentration of metabolic waste products, lactate formation-induced pH changes and mechanical shear stress. To overcome and improve preparedness for such challenges the human body adapts, in a sports-specific manner, for example by increasing red blood cell count, mitochondrial content in skeletal muscle, improved temperature management, optimized circulation, increased vascularization or structural changes in muscle, tendons and bone and increased ability to develop force in skeletal muscle. The broad range of exercise specific adaptations reflects a high degree of general interconnection between different organs and tissue. The individual elements of these interconnections could be seen as a network of well-tuned subunits in the machinery of the human body. The idea of such a complex network can help improve understanding of how different components support each other and, in a healthy body, could prevent extensive deterioration following disuse, sudden onset disease conditions or severe local injury. The adaptations or elements of such a functional network are partly structural, for example increased density of cellular organelles such as mitochondria, formation of new capillaries or more red blood cells or growth of individual myocytes. Exercise-induced adaptation can also include a mechanism for the body of these previously trained athletes to faster recover from periods of no training using what has commonly been called “muscle memory”. A mechanism for such a memory of exercise was first credited to CNS learning capacity[8], later associated with the resistance of myonuclei acquired by overload exercise to atrophy[9] and most recently has been investigated in connection with epigenetic modifications[7,10]. However, to what extent and how exactly a presumed muscle memory would work is still unknown, even its mere existence is to some extent still contested with evidence for and against it being published recently[10,11]. Concerning a general “memory” effect of training the idea of a more systemic approach to the question of its existence, consisting of multiple specific molecular mechanisms rather than one could be more compelling than previous explanation attempts and might help grasp the whole extent of such a potential memory and the resilience of long-term trained athletes to detraining periods. The two general mechanisms, the translation of the exercise-related signals into molecular responses and their subsequent adaptation effect, and the specific way these signals might be remembered on a molecular, but also structural level such as myonuclear addition or mitochondrial density could contribute to the development of an elite athlete with outstanding, sports-

specific physical abilities over time. While the difference that life-long high-level training makes for physical output is well known and separates world-class elite athletes from average individuals, the molecular effects of such life-long training are still not entirely understood. To this end, this thesis seeks to add to the knowledge of these molecular mechanisms and their consequences in high-level athletes and the way their transcriptomic and metabolomic machinery and immune system can contribute to their performance potential.

1.1 ORGANIZATION OF SKELETAL MUSCLE

The tissue enabling the active movement of the body is skeletal muscle and as such often in the focus in an exercise physiological research context. Skeletal muscle makes up 35-45% of total body mass, depending on age and sex but can be increased to over 50% depending on individual constitution and training status[12]. Anatomically, a skeletal muscle is made up of bundles of muscle fibers called fascicles (Fig. 1)[13]. Muscle fibers contain myofibrils, which are a chain of repeating functional contractile units called sarcomeres. Each level of these bundles is surrounded by a layer of connective tissue called fascia, the whole muscle by the epimysium, the fascicles by the perimysium and the muscle fiber by the endomysium. Sarcomeres consist of actin and myosin, molecular level structures that use biochemical mechanisms to shorten and lengthen, which over the length of a whole myofibril can result in substantial dimensional changes. Working together, the whole cross-sectional area of a muscle consists of numerous of contracting myofibrils, combining their individual contraction to the force production of the whole muscle. Muscle fibers can differ in their performance capabilities based on their molecular makeup and are in human roughly classified into type I, type IIa and type IIx fibers. Type I fibers are slow in contraction speed, have a smaller cross-sectional area (CSA) and therefore lower total contractile potential but are highly resistant to fatigue due to their

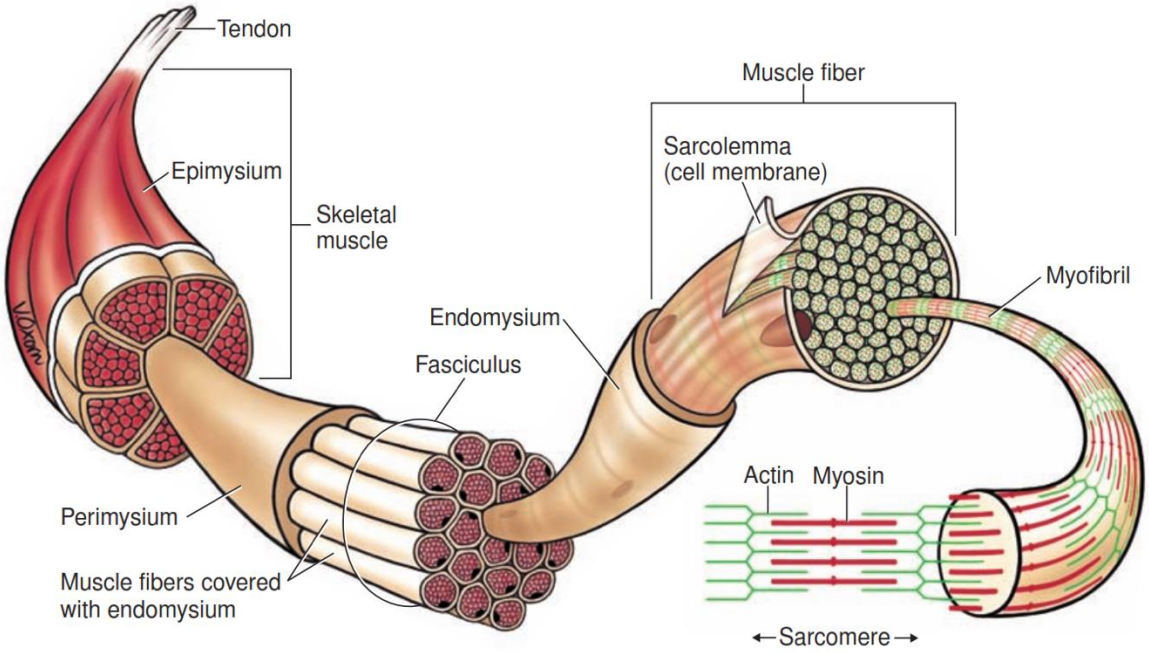

Fig. 1: Organization and structure of skeletal muscle. Individual myofibrils are bundled into muscle fibers. These fibers are combined into fasciculi of which several make up a skeletal muscle. Each is covered by a layer of connective tissue – endomysium, perimysium and epimysium. The sarcomere is the functional contractile unit composed of actin and myosin that enables the contraction of the muscle. (from [13])

high oxidative capacity. Type IIx fibers on the other hand are, have a larger CSA and higher specific force and therefore have a high contractile potential but in turn have reduced resistance to fatigue because of their lower oxidative capacity. On these performance spectra, type IIa fibers can be placed in between type I and type IIx. The above-mentioned contractile potential based on CSA and actin and myosin interaction is ATP-dependent. To produce ATP, skeletal muscle contains energy rich substrate storages in form of glycogen or lipid droplets and can import glucose and fatty acids from circulating blood. Highly trained individuals have higher storage levels and substrate uptake capacity than untrained persons. Additionally, skeletal muscles also contain other cell types than myocytes, including capillaries, adipocytes, fibroblasts, infiltrating leukocytes and motor and sensory nerves. Finally, the skeletal muscle stem cells, the – satellite cells, provide regenerative and proliferative potential. Together, the structures of the skeletal muscle contribute in different ways to the transcriptome, proteome and metabolome of the body and the different layers mutually influence and regulate each other in a complex manner (Fig. 2)[14].

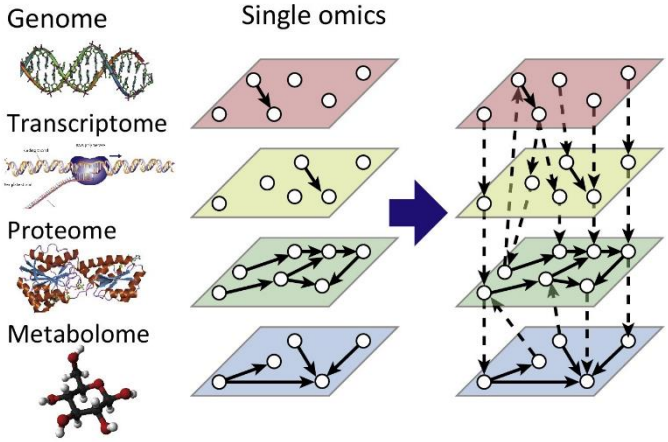

Fig. 2: Different layers of molecular -omics and their mutual influence within and between layers of -omics. (modified from [14])

1.2 STARS – A PATHWAY FOR MOLECULAR MECHANO-TRANSDUCTION OF EXERCISE SIGNALING

Adaptation to regular exercise training is based on molecular processes triggered by a number of exercise-related physiological changes. As an example, acute exercise requires an increased supply of oxygen to the active tissue skeletal muscle which, when reaching intensity limits, might not be able to be satisfied. Local hypoxia, and other signals, triggers the expression of vascular-endothelial growth factors that stimulate angiogenesis[15–19]. Another example could be the induction of micro-trauma and damage to individual muscle fibers that activate local inflammation and a regeneration process during which damaged muscle fibers are repaired and restored with increased strength and structural integrity as a consequence[20–22]. Such a process partly depends on the efficiency and the flexibility of the immune system, which is strongly connected to exercise and also long-term adaptation to exercise[23]. An even more direct pathway for the molecular mechano-transduction of an exercise impulse is the STARS-pathway, which is centered around the protein striated activator of rho signaling (STARS)[24,25]. The STARS protein can be found in striated muscle such as skeletal and heart muscle and is localized to actin-binding regions in the sarcomere[25]. Efficient contraction of skeletal muscle requires a high organizational level of actin to form the contractile unit of the sarcomere. Structured, polymeric F-actin is formed from monomeric G-actin with the help of amongst others STARS, which facilitates such a polymerization process[25]. The monomeric G-actin pool exerts a suppressive effect on the activity of the serum response factor (SRF; Fig. 3), a transcription factor

important in the intracellular transduction of mechanical signals with downstream targets such as the myogenic regulation factor MyoD[26], the myocyte enhancer factor-2 (MEF2)[27], but also the fat oxidation enzyme carnitine palmitoyltransferase-1β (CPT-1β) and transcription factors such as JunB influencing muscle growth regulation[28]. The suppressive effect of G-actin on SRF transcriptional activity is mediated through myocardin-related transcription factor-A (MRTF-A), which is required for SRF transcriptional activity. G-actin prevents the translocation of MRTF-A from the cytoplasm to the nucleus, but the decrease of the size of the G-actin pool during the polymerization effect allows for a nuclear translocation and subsequent increased SRF activity[24,29]. This molecular mechanism has to a certain extent been shown to be self-regulatory, as both SRF and its transcription products can act as transcriptional regulators of STARS itself eliciting a feedback-loop mechanism[30]. However, another key regulator of STARS is a transcription factor complex formed by the estrogen related receptor alpha (ERRα) and the peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1α), both master regulators in the context of exercise[31–37]. Both have been previously shown be influenced by physical activity, to regulate genes involved in skeletal muscle function and to directly interact with the regulatory region of the STARS gene[28,38–40]. PGC-1α and ERRα are expressed to a variable degree depending exercise parameters such as type of exercise, intensity, duration, frequency but their expression also differs between muscle fiber types[41,42]. These parameters as well as muscle fiber composition is highly relevant in the context of the specialization to different kinds of sports[43–46]. Other previously published studies showed clear differences in PGC-1α and ERRα expression based on specific training programs and in response to short- or long-term training[28,47–51]. In the context of the present thesis and its focus on life-long trained athletes it is also interesting to mention that the acute increase of PGC-1α expression following each bout of exercise has been shown to be significantly decreased over time, which considering that STARS is under the control of PGC-1α could imply a decreased stimulation of the STARS pathway over the course of an athletic career[52]. The differential response of STARS to different kinds of exercise has been demonstrated in several previous publications[28,53,54]. Together, these genes – the PGC-1α/ERRα complex, STARS, MRTF-A and SRF - can be seen as an extended STARS pathway (see **paper I** Figure 6), anchored in one of the master regulators of exercise (PGC-1α), connecting to specific downstream regulatory elements

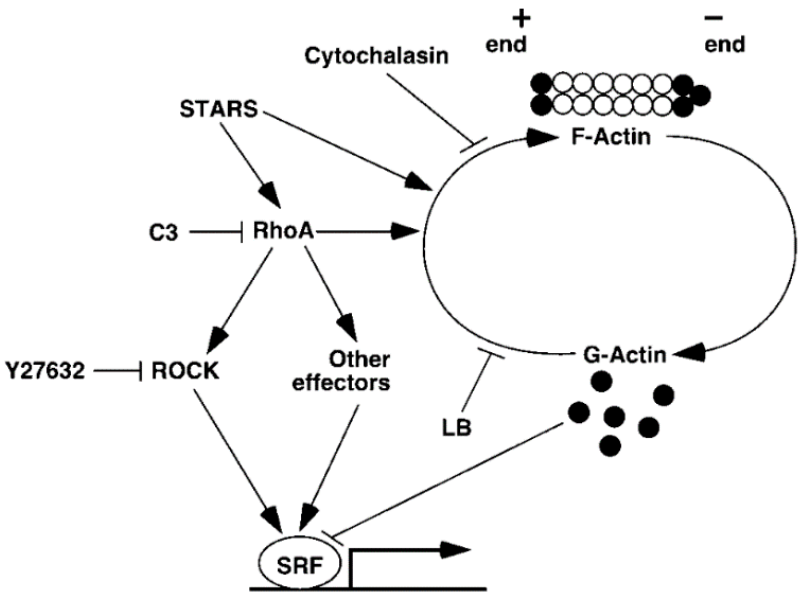

Fig. 3: The STARS molecular context. STARS promotes the formation of structured polymeric F-actin from pools of monomeric G-actin effectively decreasing the size of the G-actin pool. The suppressive effect of the G-actin pool on the activity of transcription factor SRF is therefore decreased by STARS activity which increases transcription of SRF target genes. (from [25])

(MyoD, MEF2, JunB). The connecting role and potential dependence on type and consistency of exercise of the STARS pathway makes it an interesting element of the molecular mechano-transduction of exercise signaling for life-long high-level athletes.

1.3 EXERCISE MEMORY

The adaptations that are induced via different pathways manifest as physical changes of the body on subcellular, cellular, tissue and whole-body levels. Retaining such adaptations – for example increased muscle mass and stroke volume from the athletic perspective is key to subsequent further improvement but can also be vital to health and quality of life with progressing age[55]. To this end, it has previously been reported that individuals with a history of strength training are able to come back from a period of no or limited training faster or to a greater extent suggesting that skeletal muscle in some way could possess a "cellular memory" of its training history[10,56,57]. But what exactly such a muscle memory might be and how it would work has been the subject of recent research efforts focusing on specific molecular mechanisms[10,11]. As previously mentioned, taking a step back and looking at the whole system of the biological machinery of an exercising body, it becomes quite clear that large interdependencies exist between individual organs and tissues. Large structural adaptations require the maintenance of other supporting tissue, for example increased muscle mass would require or otherwise be limited by vascularization processes to supply the increased muscle mass with oxygen, nutrients and other metabolites such as energy rich substrates[58]. The increased force that is developed by such increased mass requires strengthened tendons to translate the muscles' full potential into movements. The immune system can be influenced by cytokines expressed by skeletal muscle in connection with exercise, but organs of the immune system also influence and give rise to immune cells that can have an effect on the muscle. The efficiency and performance of ventilation, the uptake of oxygen via the respiratory system is a limiting factor in over-all oxygen availability and as such sets a limit for physical performance[59]. Apart from the interdependencies mentioned above, further examples exist that can be especially interesting in the context of exercise and undergo adaptation in response to life-long exercise. Through the interdependencies mentioned above, such adaptations partially build mutually on their improvement and by doing so could create a structural safety net that could be regarded as a memory effect by providing increased resilience to detraining. In skeletal muscle, acute resistance exercise in skeletal muscle is associated with increased expression of genes and proteins related to myogenesis, angiogenesis and structural elements of the muscle such as the extracellular matrix[60]. One limiting factor in such a process is the potential transcriptional output of myonuclei, a capacity that is increased through a process called myonuclear addition in which satellite cells fuse with the muscle fiber and add their nucleus and with that their transcriptional capacity to the fiber[61]. Retaining such increased capacity during periods when no training occurs could result in the observed faster recovery process in previously trained individuals. Much attention has been given to a proposed mechanism of myonuclear addition via satellite cells[9,62,63], however with current specific evidence of such a process limited to animal models only[64]. The existence of an influence of long-term training on the molecular level, for example

on gene and protein expression in human has been shown in numerous studies, demonstrating attenuating but also sensitizing effects[36,49,52,57,65–69]. A recent paper showed resistance-training induced epigenetic modifications to be sustained after a 7-week period of detraining in humans, a modification process that could partially explain differences in transcription rate and recovery[10]. In contrast, a recent paper from our own lab showed that previous endurance training does not result in transcriptomic differences upon retraining following a 40-week period of inactivity[11]. Those results would suggest a more complex underlying mechanism, that could depend on the type of exercise performed but also on the duration of the detraining period. The sparsity of scientific evidence for a specific molecular mechanism in humans warrants further investigations to reach a firmer conclusion on the tentative existence of and mechanisms underlying muscle memory.

1.4 LONG-TERM HIGH-LEVEL TRAINING AND LARGE-SCALE DATA

Over a long-time period, consistent and systematic exercise training can result in highly specialized high-level athletic performance[70]. The specific adaptations depend on the performed exercise type spanning a continuum between endurance and resistance type training. The specific adaptations are partly due to transient changes in gene expression following each exercise bout. Endurance efforts are largely limited by the ability to produce ATP through oxidative phosphorylation which depends on substrate availability and gas exchange. Accordingly, physiological adaptations include improved cardiac output, increased absolute numbers of red blood cells, improved vascularization and perfusion of peripheral tissues, increased mitochondrial function and density and improved efficiency of energy converting processes and metabolic flexibility[71–75]. Physical performance in resistance-based sports is largely limited by contractile force and short-term energy supply to the sarcomeres. Adaptations include the reinforcement of structures and processes that improves force output - increased CSA by skeletal muscle hypertrophy as well as improved neuromuscular control with increased recruitment of motor units, increased per-area force production by enhanced muscle fiber activation and biomechanically more beneficial movement patterns[76–78]. As a consequence, life-long exercise training specialization results in two largely different kinds of athletes, one capable of long duration physical efforts requiring high oxygen uptake and lactate threshold and one capable of short duration high power output requiring a large CSA, and superior neuromuscular control. Furthermore, both forms of lifelong exercise training can induce some alterations in fiber type composition and improve metabolic control[1–3], delay general aging effects and lower the risk for cardiovascular disease, cancer and osteoporosis[4–6].

Some of these adaptations might be beneficial for both endurance and resistance based physical efforts. As an example, a limiting factor for an increase in muscle mass could be vascularization, as a certain level of capillary density is required to support muscle tissue[79,80]. Increased mitochondrial content may provide large amounts of energy but endurance performance still depends on a certain level of muscular strength. Biologically, both examples might be indicators of the potential of incorporating a certain level of concurrent training in a training routine, increasing capillary density through endurance exercise to overcome limitations in muscle mass increase or increasing muscle mass to utilize the large energy production capacity. Other adaptations are furthermore common to

both exercise modalities, for example increased insulin sensitivity in endurance athletes is a consequence of improved mitochondrial function and a consequence of increased muscle mass in strength athletes[81]. Both specializations can result in a shift in muscle fiber type composition, specifically from type IIx to type IIa, and in increased local glycogen storage capabilities[81,82]. Together, such adaptations make specialized athletes largely different from untrained people even in a non-training context. As previously mentioned, besides the performance aspect both forms of exercise have numerous health benefits for example in cardiovascular[83] and metabolic diseases such as type 2 diabetes[84,85], cancer[86,87] and osteoporosis[88,89] resulting in an increased quality of life and a lengthened lifespan[6,90]. While the effects of acute and medium-term exercise are extensively studied, the molecular long-term impact of high-level training is less understood. This is rooted at least in part in the logistic and organizational complexity of decades-spanning cohort or even intervention studies, even though some research group's attempts at similar designs are currently ongoing. To overcome the practical limitations due to the experimental design, researchers have employed animal models to address the question of life-long exercise effects. While such models do have their benefits in allowing for a complex, multi-level experimental design, it should be kept in mind that their results can only be used as tentative indicators of the human response to exercise and require extensive translational context[91]. Because of the limitations of animal models, a retrospective approach in the design of human studies can be a good choice to improve mechanistic knowledge of life-spanning adaptation processes, with the potential to uncover differences between untrained, endurance trained and resistance trained individuals, which might improve understanding of fundamental mechanisms and help treat some of the diseases mentioned above.

1.4.1 Sex differences

Besides the difference between trained and untrained individuals, sex is an often-overlooked factor that should be considered in the context of exercise physiology. Not only regarding general sex-based differences in the response to exercise but also the above introduced idea of a functional systemic memory of exercise would reinforce the significance of sex difference, considering that all organs of the body are highly interconnected and influence each other's function. When focusing on skeletal muscle alone there might not be major sex differences. However, considering the large hormonal difference between females and males, there might be structural and functional differences in other organs and tissues which might play a role in a "functional network memory" in the context of exercise. It should be noted, not only historically but still today, that most of the research in exercise physiology has been performed with male subjects. Sex-specific differences, regardless of their magnitude, are frequently not considered in experimental design, despite some previous publications showing notable difference between male and female elite cyclists[92,93] and large numbers of differentially expressed genes between men and women at rest[94]. Considering the biological differences between male and female physiology, an increasingly precise and personalized approach to understanding performance physiology therefore has to include comparisons and attempt to differentiate between the sexes.

1.4.2 RNA sequencing

To study the transcriptome, large-scale data approaches such as RNA sequencing have shown to be very useful for obtaining both an overview over general tendencies in gene expression but also for identifying individual genes in the context of their specific pathway or functional context. In a number of studies, global gene expression methods have been employed to identify mechanisms involved in the adaptation to physical activity[94–98], though none of those in a long-term training context. While acute and medium-term effects can have some influence on physical performance capacity and health status, it is over a longer term that transient changes in the transcriptome following each training session as well as gradual shifts in the transcriptome unfold their extensive impact.

1.4.3 Genome-scale metabolic models

While transcriptomic information is valuable but has to be interpreted in a functional context[99,100], the local availability of metabolites might play a large role for factual performance in an exercise context. However, metabolites are product and fuel of enzymatic activity which is steered amongst others by gene expression. Previously, large databases mapping gene-enzyme-metabolite interconnections have been created with the Human Protein Atlas[101,102] and the Metabolic Atlas[103]. Based on the molecular contextual information of metabolites from both sources, genome-scale metabolic models (GEMs) were developed. GEMs can be fed with transcriptomic data and, using the contextual information and metabolic equations, provide predicted metabolic fluxes and metabolomics as an output (Fig. 4)[104–108]. Consequently, gene expression data can be used to infer the "metabolic status" of entire sub-cellular compartments. While such an approach for *in silico* metabolomics is based on transcriptomic data, bioinformatically adding a layer of information from GEMs allows for a look at transcriptomic data through the prism of metabolic context uncovering patterns in gene expression not visible before as shown in previous publications[105,108,109]. For the understanding of metabolite-dependent physical performance potential this particular tool can add great value and context to transcriptomic data[108,109]. This can aid in the understanding of not only limitations of physical performance but also, by identifying crucial gene networks connected to pathological alterations in metabolite availability in diseases states lay the foundation for adequate interventions and therapies.

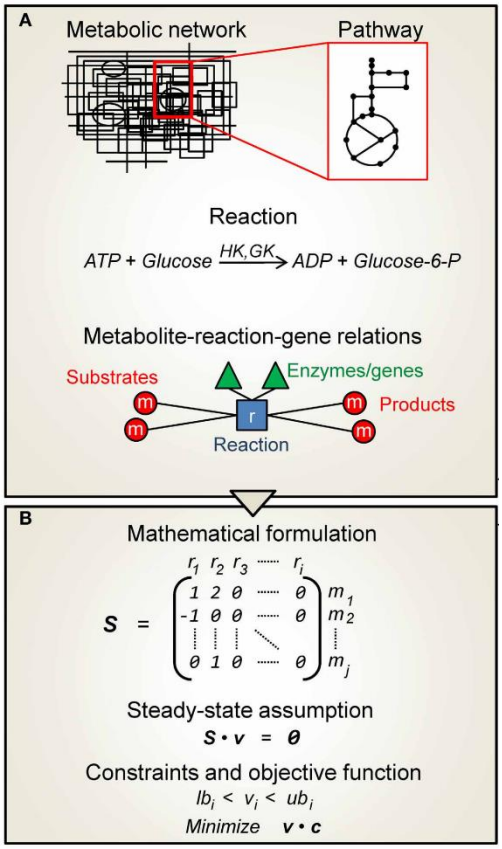

Fig. 4: Principle of genome-scale metabolic modelling. Based on pathway and metabolite reaction formulas mathematical models can predict metabolites. (from [108])

1.4.4 Motif activity response analysis

In a similar way to the use of GEMs to infer metabolomics from transcriptomics, motif activity response analysis (MARA) can be used to infer transcription factor motif activity based on transcriptomics. Transcription factors regulate gene activity and by doing so influence metabolite concentration. Transcription factors bind to motifs in the promoter or regulatory sequence associated with the transcription start site of each gene and can regulate the expression of the associated gene. Some of these motifs are highly specific for individual transcription factors while others can bind a whole family of transcription factors[110]. Together with transcriptional co-factors, promoters, enhancers, silencers and other gen-regulatory mechanisms, transcription factors make up the so called "regulome", which summarizes all processes and elements that regulate gene expression. As in GEMs, an added layer of regulomic information in the form of transcription factor-motif interaction can make functional, context-specific networks and gene connections visible that cannot be seen using transcriptomic data only[111,112]. A database of the regulomics layer of information has previously been created by the FANTOM (functional annotation of the mammalian genome) consortium, which has published promoter-based expression profiles of mammalian cell-type-specific transcriptomes with the FANTOM5 project[113]. The FANTOM5 database includes the mapping of the regulatory region of genes and the identification of transcription factor-binding motifs that can be used for the for the analysis of skeletal muscle tissue. Combining functional information from FANTOM5 and gene expression data from RNA sequencing, the recently developed MARA[114] can provide a comprehensive overview of the regulatory network following acute exercise in life-long trained athletes. This predictive method works by inferring motif activity based on the expression of genes that contain conserved instances of the transcription factor motif in their core promoter regions[110,114]. This is achieved by a catalogue of motifs and the strength of their link to associated genes and the expression levels of these genes which is used to quantify a motif activity score. However, by itself MARA, like GEM, cannot prove causality between the motif itself and a gene but motif activity calculated on the basis of the expression of their member genes has previously been shown to accurately predict transcriptional networks in contexts such as cellular differentiation[111]. Taken together, MARA could be taken as a suitable indicator of transcription factor and network activity when combined with functional evidence of regulatory linkage such as known biological mechanisms.

1.5 ACUTE EXERCISE PERFORMANCE OF HIGH-LEVEL ATHLETES

Many of the adaptations following long-term high-level training become more obvious during acute exercise than at rest. Acute exercise has a broad effect on the human body and amongst others influences the circulatory system, thermoregulation, substrate availability and hormonal signalling of organs involved in or affected by the activity. Physiologically, comparing the performance output of high-level athletes to untrained individuals, they are able to run or cycle faster and fatigue later, or lift heavier and for a longer period. Differences in performance output are based on underlying specifically adapted molecular processes, such as metabolic processes that emerge during acute exercise. Previously conducted studies showed that specific gene expression responses to exercise

decreased in their amplitude during progression through subsequent sessions, indicating an influence of training history on acute molecular mechanisms[52,97,115,116]. However, while aforementioned studies have investigated gene expression following acute exercise or following regular exercise training over weeks or months[52,117–119], it remains unclear how gene expression following an acute exercise bout is influenced by a history of high-level training over many years. Increasing the understanding of how high-level physical performance works and what makes it different from physical efforts of untrained individuals on a molecular level could provide valuable information about performance-limiting processes and potentially influence the optimization of training protocols to focus on training modalities that trigger and train key mechanisms. An increased understanding might also aid in the development of treatments and prevention strategies of diseases, especially in situations where traditional exercise cannot be utilized.

The complexity of the molecular response to acute exercise on different omics-levels but also the potential that lies in the integration of multi-level analyses has recently been demonstrated by the identification of novel biological pathways involved in the response to acute endurance exercise through transcriptomic, proteomic and metabolomic analysis from blood samples of healthy volunteers[120]. Due to the complexity of acute intervention studies often involving several series of timepoints, results from multi-level analyses are sparse, even more so in study designs of higher complexity involving different subject groups and forms of acute intervention. Additionally, the recruitment of life-long trained high-level athletes to potentially invasive intervention studies might pose an organizational problem. Another interesting question would be the investigation of the impact of concurrent endurance and resistance training on physical performance. However, to this date only limited knowledge is available if and how highly specialized athletes respond differently to a form of exercise they are not specifically adapted to. Such knowledge would be required and form the basis of a potential future investigation on how concurrent training can be utilized of performance improvement, however there are indications of both beneficial and detrimental effects[121–125]. This is especially true for the molecular effects of such unfamiliar exercise as the mentioned existing studies rather focus on physiological performance effects. Taken together, the notion that the acute response to endurance or resistance training in long-term adapted athletes would be exercise modality- and possibly exercise background-specific seems increasingly likely[97,116].

1.5.1 Acute exercise and the immune system

Apart from the previously mentioned impact on the physiology of the exercising individual, acute exercise can influence the activation state of the immune system, in particular the number or activation status of circulating immune cells. Furthermore, acute exercise can have effects on inflammatory processes and gene expression in immune system target tissues[126]. A direct influence on inflammatory status in skeletal muscle could make a big difference for speed of recovery from acute exercise. Furthermore, via its immunomodulatory effect skeletal muscle itself has been shown to have the potential to influence inflammation beyond acute exercise[127]. Through that mechanism, the immune system via skeletal muscle can have the ability to permanently influence the whole body

which in turn responds and adapts accordingly. Amongst others the inflammatory and immune system response can lay a foundation beneficial for health, acute performance and to a certain extent guide and influence the body's adaptation to exercise. Immune cells can be separated into two groups by their hematopoietic origin from either a myeloid or a lymphoid progenitor cell (Fig. 5)[128]. Monocytes, macrophages and granulocytes originate from a myeloid progenitor cells and are part of the innate immune system. T-cells, B-cells and natural killer (NK) cells originate from a lymphoid progenitor cell and are part the adaptive immune system, with the exception of the NK cells which to a certain extent possess the characteristics of both the innate and the adaptive immune systems. Furthermore, the innate and the adaptive immune systems function on different basic mechanisms. While the adaptive immune system relies on antigen presentation, specific pattern recognition and the development of an immunological memory that enable a highly specific targeted immune response, the innate immune system is broader in its effect and acts by attacking and removing cells damaged or foreign to the body. Depending on their origin, the two categories of immune cells depend on different mechanisms to accomplish immune defence. Myeloid origin immune cells rely on phagocytosis and cell toxic substances and agents which are largely used to eliminate cells that are damaged or express apoptotic signals. Lymphoid origin NK-cells are more specific, and attack compromised host cells via a prior surface pattern recognition. They can be separated into two subsets, the mature CD56+^{dim} NK-cells that exhibit high migratory potential[129] and the CD56+^{bright} NK cells that

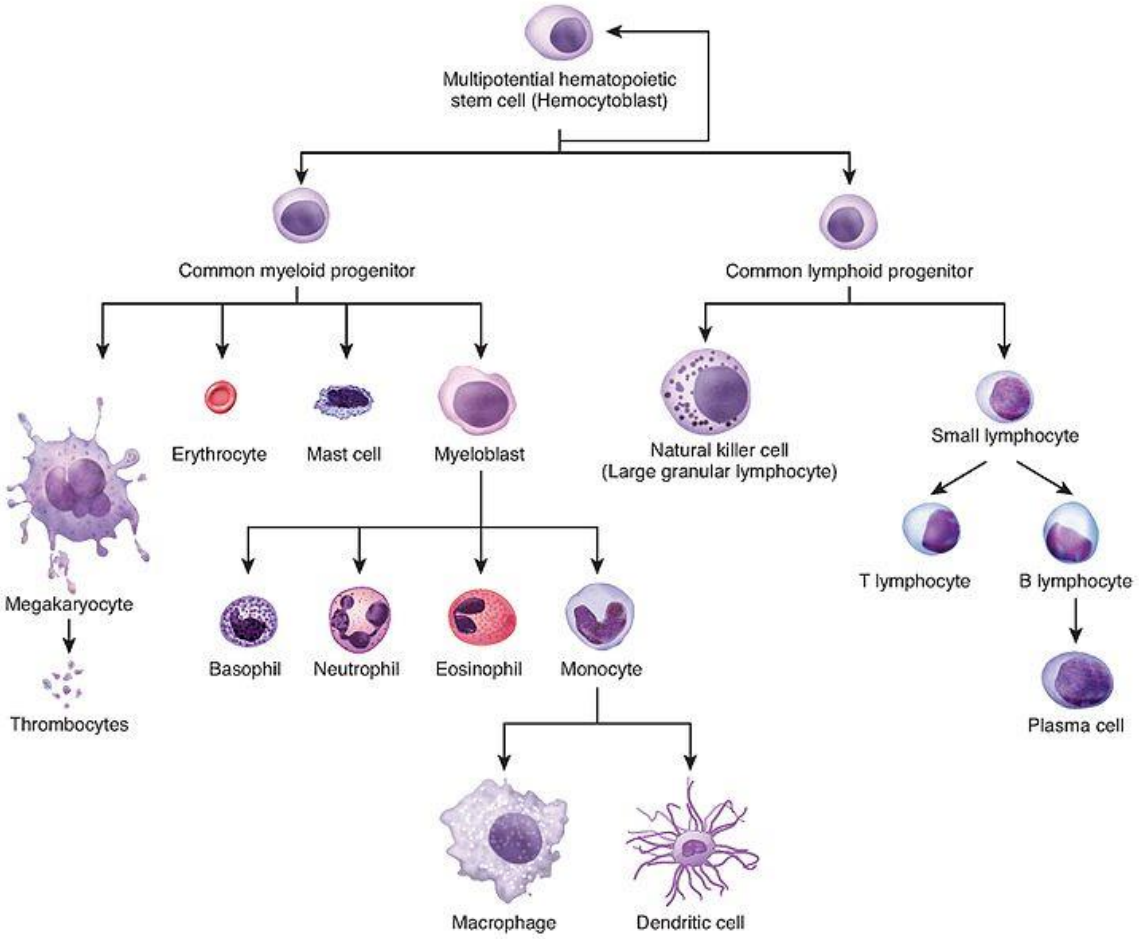

Fig. 5: The hematopoietic origin of blood cells. The myeloid progenitor cell (left branch) gives rise to cells of the innate immune system, including monocytes, macrophages and granulocytes. The lymphoid progenitor cell (right branch) gives rise mainly to cells of the adaptive immune system such as T-cells and B-cells. (from [128])

reside in secondary lymphoid organs[130,131]. The lymphoid T-cells are part of the adaptive immune system. Their cytotoxic version, CD8+ T-cells effectively induce apoptosis in target cells identified through specific antigens while CD4+ T-cells moderate the immune response by contributing to the maturation but also the shutdown of other immune cells. Furthermore, B-cells that produce target specific antibodies are part of the adaptive immune system and are triggered to produce such antibodies by T-cells when they encounter a specific antigen, the very definition of adaptivity. Finally, TCRγδ+ T-cells are a mix of both systems, capable of innate-like pattern recognition and adaptive-like targeted effect on memorized specific antigens.

The complementary effect of both the innate and the adaptive immune system and their cells contribute to physical performance capacity changes by alterations in inflammation, defence and regeneration processes in their target tissues[132,133]. Exercise parameters such as intensity, duration and frequency or muscle mass activated modulate the immune response, parameters which are all influenced by training status of the individual. In turn, continued, form of exercise-specific activation of the immune system will shape the immune system itself over time, not least in muscle tissue being a host of for example resident macrophages, or by increased turnover of immune cells and immune system stem cells[127,134]. The role of skeletal muscle as a "motor" of the immune response and its contribution to the shape of the immune response to exercise make it plausible that life-long training influences the immune system response to acute physical exercise. The acute immune response is, besides the activity of local resident immune cells, largely mediated through blood-borne immune cells circulating through the body, which makes such changes readily observable through immune cells circulating in blood. Immune cells originate from bone marrow and thymus, reside largely in lymph nodes and the spleen and are recruited to the circulation from the extra-vascular storage depots. This can be effected by cytokines originating from skeletal muscle during exercise, but also by mechanisms that increase blood pressure via adrenergic stimulation[135–137], showing that the role of the circulatory system extends beyond being a mere mode of transport. Immune cell populations delivered to the target organs influence local inflammation and immune system response. Such a mechanism can modify the expression of cytokines resulting in a feedback loop-like process with the potential to influence the whole body[138].

While skeletal muscle is a major immune cell infiltration target organ in connection with physical exercise it also holds substantial reservoirs of resident immune cells that are activated[132,133,139] and then affect subsequent phases of local inflammation, defence processes and regeneration[140]. The size of the contribution of such local reservoirs to the total number of cells depends on the immune cell type. For macrophages, a major player in the immediate, innate, inflammatory response in skeletal muscle, the local reservoir can be the source of the majority of active immune cells in the tissue[139]. Immune cells, following tissue infiltration or local activation of resident immune cells, influence and trigger immune cell-specific local gene expression. Such genes are not only part of the local immune response but through their influence on muscle performance, gene expression and muscle-originating exercise signals would contribute to the system-wide network of mutual exercise-induced influence of organs and tissues. Over time, the repeated modulation contributes to a

multitude of mechanisms that are integral to the reshaping of skeletal muscle tissue directly and other tissues of such a network indirectly in consistently training athletes[141]. This primarily local response will then lead to long-term adaptation processes such as increased vascularization of the muscle resulting in improved distribution of oxygen, nutrients and also immune cells to that tissue. Removing limiting factors to exercise performance will again improve that tissues capacity to respond to acute exercise with improved performance, continuing the cycle of mutual influence[142]. The mentioned examples demonstrate the close crosstalk between skeletal muscle, the mechanically active organ in exercise and the cardiovascular and nervous systems and tissues such as bone marrow, thymus, spleen and lymph nodes and tonsils that produce and store immune cells[143]. Apart from classical immune tissues such as spleen and tonsils such reservoirs can also be found in the marginal zone of the vascular system[144] and the lungs[145], organs that will experience increased perfusion during acute exercise and therefore can contribute more of their reservoirs content. Such a systemic perspective, and insight into the influence of a life-long high-level history of targeted endurance- or resistance-based training on the immune system contribution to such a network is sparse. Previous studies have focussed on investigating acute exercise, but baseline differences between leukocyte populations remain understudied. Knowledge about the influence of a high-level exercise background on the acute immune system response as a result of a network-modulation of the immune system would equally be of high value to understand such a mechanistic network in the context of life-long specific adaptation. As previously mentioned, perceived exertion and the intensity of the specific acute exercise can modulate the shape of the immune response. It might therefore also be interesting to investigate how high-level athletes would respond to an acute session with a type of exercise that they are not used to, for example strength athletes conducting a session of endurance exercise. It would seem likely that life-long strength athletes, not routinely challenged with intense endurance efforts, show a different immune response to endurance than to resistance exercise, and endurance athletes in a similar way when doing resistance exercise considering the unfamiliarity of the performance demands. However, no study to date has investigated such a mechanism. For example, high level of exertion could potentially lead to an initial “high-stress” inflammatory response to an unfamiliar form of acute exercise that subsequently subsides into a more coordinated immune response that could be positively affected by any kind of previous exercise experience that give the immune system and the whole body network of exercise adapted organs and tissues a higher level of flexibility. Considering the idea of a network of crosstalk would make an investigation of cross-over responses of circulating immune cells and skeletal muscle-based gene expression an even more valuable addition to our current understanding of whole-body exercise adaptation in general and the immune system of the exercising athlete in particular.

2 AIMS

The overall aim of this thesis was to investigate molecular mechanisms that support and maintain adaptations to life-long high-level exercise training.

The specific aims were to:

Investigate the effect of an acute bout of exercise and 12 weeks of endurance training on gene and protein expression of factors in the STARS pathway in human skeletal muscle (**Paper I**).

Identify how skeletal muscle responds differently to a period of training when it has been trained before compared to having no prior history of training and explore potential gene expression and epigenetic mechanisms contributing to such an adaptation (**Paper II**).

Study the effect of life-long high-level exercise training in strength- or endurance-based sports on histological structure of skeletal muscle and gene expression at baseline and investigate how such an adaptation might be useful in the context of disease prevention and treatment (**Paper III**).

Study the effect of acute endurance and resistance exercise on global gene expression in skeletal muscle of life-long trained individuals with strength- or endurance-based background and bioinformatically investigate the related regulomic and metabolomic networks (**Paper IV**).

Investigate which effects acute exercise has on the immune cell populations in circulating plasma, how different strength- or endurance-based backgrounds influence such a response and which effect these immune cells have on gene expression in skeletal muscle as a target tissue (**Paper V**).

Taken together, these aims have the purpose to investigate the transcriptomic, metabolomic, regulomic and immune system consequences of life-long high-level endurance and resistance training and contributing molecular mechanisms.

3 METHODOLOGY AND STUDY DESIGN

3.1 STUDY LAYOUTS AND METHODS OVERVIEW

The studies for **papers I-V** were designed as human exercise intervention and cross-sectional studies. **Paper I** is based on two intervention studies, one consisting of acute exercise, the other one of long-term training. In the acute study, 20 male and female subjects were included after undergoing VO_2-peak testing (inclusion criteria: <60ml·kg^{-1}·min^{-1} and <50ml·kg^{-1}·min^{-1}, respectively). Then they performed a 60-minute cycling session on a stationary bike at 50% (first 20 minutes) and 65% (remaining 40 minutes) of their VO_2-peak. Biopsies from M. vastus lateralis were taken at five timepoints around the intervention (Fig. 6A). In the long-term training study, 23 male and female subjects were recruited and included using the same inclusion criteria as in the acute study and progressively trained a randomized leg by one-legged knee extension exercise four times per week for 12 weeks. Muscle biopsies from *M. vastus lateralis* were taken before and after the intervention period (Fig. 6B). In both studies, food intake and circadian effects were controlled for by standardized meals, meal diaries and consistent time plans throughout the experiment. For the long-term unilateral training study of **paper II**, 19 healthy male and female subjects were recruited that have never been engaged in any systematic sport or physical activity. The subjects were subjected to a 10-weeks undulating, unilateral leg strength training program that consisted of three times per week leg press and leg extension training at moderate (70-75% 1RM) and heavy loading (80-85% 1RM) and low-load blood flow restriction training in week 4 and 8 to maximize hypertrophy (Fig. 7). Additionally, subjects consumed 25g whey protein after each training session. Following the training period, subjects completed 20 weeks of detraining in which no training was allowed. At the end of the detraining period all subjects performed one bilateral acute exercise session including leg press and leg extension (at 75% 1RM) before and 1h after which muscle biopsies were taken from *M. vastus lateralis* of both legs. More details can be found in a previously published paper[64]. **Paper III** is based on one cross-sectional study without intervention (Fig. 8A). Male and female subjects (n=40) were included after VO_2-peak and peak torque knee extension testing. All subjects were pre-screened by questionnaire and for the trained groups

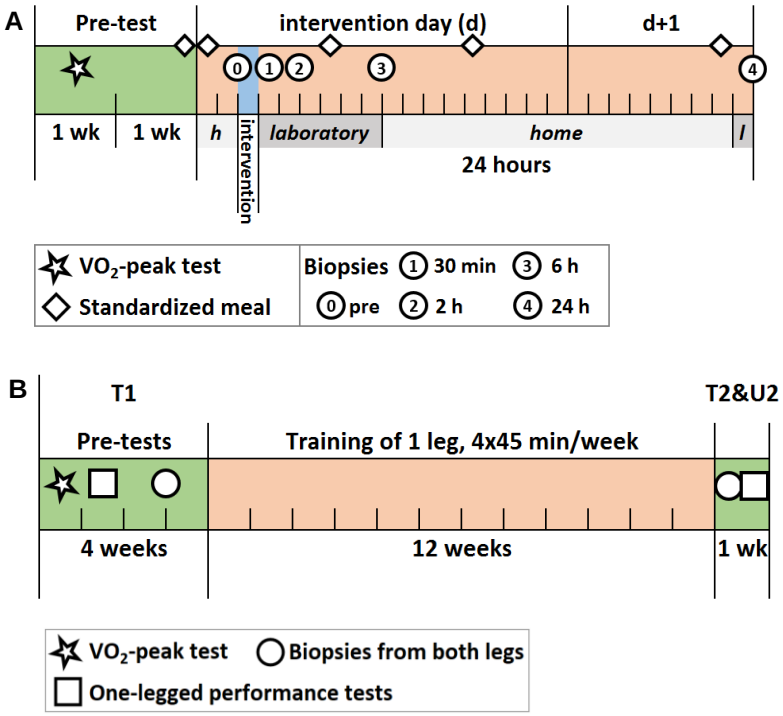

Fig. 6: Layout of acute (**A**) *and long-term* (**B**) *training part of the study that* ***paper I*** *is based on.*

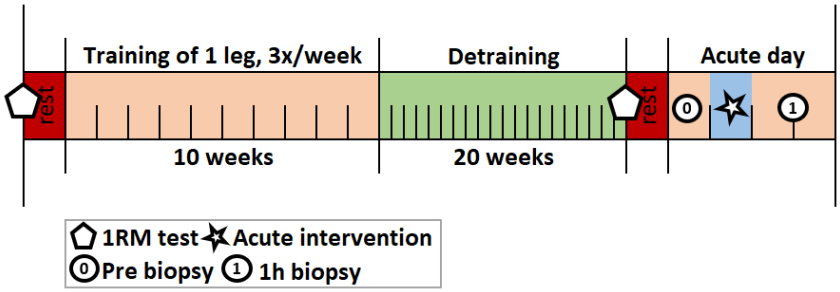

*Fig. 7: Layout of the unilateral longitudinal study **paper II** is based on.*

they were required to have at least 15 years of high-level experience in their respective strength- or endurance-based sports. Untrained control group subjects were required to have no activity of targeted exercise training. All subjects were in the age range from 33 to 52, healthy and non-smoking. For inclusion in the strength trained group subjects had to have a tested peak torque of over two standard deviations of the average of the untrained control group and a tested VO_2-peak in the 25th-75th percentile of their respective age group[146]. For inclusion in the endurance trained group subjects had to have a tested VO_2-peak >95th percentile of their respective age group and a tested peak torque lower than the lowest testing subject in the strength trained group. For inclusion in the untrained control group subjects had to have a tested VO_2-peak in the 25th-75th percentile of their respective age group and a tested peak torque of at least two standard deviations below peak torque of the strength trained group. They were then divided in three different groups: strength trained, endurance trained and untrained control. Muscle biopsies from *M. vastus lateralis* of both legs were taken from all subjects. **Papers IV-V** are based on the same acute intervention study. Twenty-four men were recruited and divided in three different groups defined in the same way as in **paper III**. Inclusion testing followed the same criteria as in **paper III**. Additionally, one repetition maximum (1RM) in knee extension exercise was assessed. All subjects completed two sessions of acute intervention, one session consisting of nine sets of eight repetitions (reps) of knee extension at 80% of their tested 1RM, one session consisting of 30 minutes of cycling on a stationary bike at

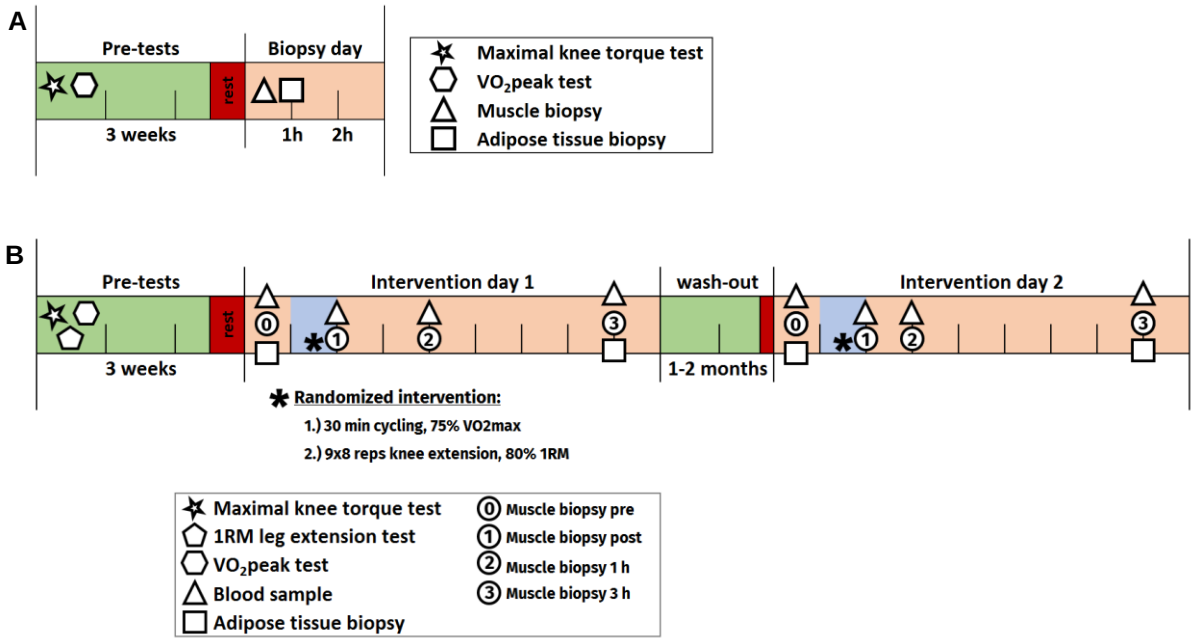

*Fig. 8: Layout of cross-sectional (**A**) and acute intervention (**B**) study with life-long trained athletes that **papers III** (**A**) and **IV-V** (**B**) are based on.*

75% of their tested VO_2-peak. Both sessions were separated from each other by one to two months and the subjects were randomized to their first acute intervention protocol. Biopsies from *M. vastus lateralis* and blood samples from *V. mediana cubiti* were taken at four timepoints around the intervention (Fig. 8B). Food intake and circadian effects were controlled for by meal diaries for dinner and a standardized breakfast before the acute intervention and consistent time plans throughout the experimental period.

In **papers I and III- V**, physiological testing for aerobic endurance was performed by a direct VO_2-peak test. **In paper II**, 1RM was measured using bilateral leg press and leg extension. For **papers III-V**, knee extensor peak torque was assessed by isokinetic unilateral knee extension test, leg extension strength by isotonic bilateral 1RM test. In **paper III**, body composition was assessed by whole body Magnetic Resonance Imaging (MRI). Muscle biopsies were analyzed histologically by immunohistochemistry (**papers I, III, IV**), for gene (qPCR in **papers I-II**, RNAseq in **papers III-V**) and protein expression (western blot in **papers I-II**), fiber type composition (MHC gel and silver staining in **paper III**, immunohistochemistry in **papers III-IV**) and for enzymatic activity (citrate synthase activity in **papers III-IV**). Additionally, for **paper II** methylation analysis was performed using pyrosequencing and for **paper V**, blood samples were analyzed by flow cytometry. In **papers III-V**, advanced bioinformatic analysis was performed including differential gene expression analysis, GO pathway analysis using REVIGO, gene set enrichment analysis using the fGSEA package, transcription factor motif activity response analysis (MARA) based on the FANTOM5 database and genome-scale metabolic modelling (GEM).

3.2 APPLICATION OF COMPARATIVE ENDURANCE AND RESISTANCE EXERCISE IN PHYSIOLOGICAL EXPERIMENTS

Comparing effects of resistance and endurance exercise in an experimental protocol design can be challenging if the goal is to in some way match the amount of exercise performed in a specific muscle. Generally speaking, one way to match exercise types would be by a factor such as overall calorie expenditure. However, this might not be satisfactory because endurance exercise affects the whole body and leads to a larger total energy consumption with a lower local muscle energy expenditure compared to calories consumed in targeted resistance exercise that has a higher local energy expenditure. Matching the different types of exercise purely based on total calories consumed would therefore require an extensive amount of resistance exercise sets to be performed.

3.2.1 Matching resistance and endurance exercise

When choosing to compare two lower limb exercises that have a similar movement pattern and both mainly recruiting the *M. quadriceps femoris* activity – cycling for endurance exercise and knee extension for resistance exercise, reasonable matching can be achieved based on caloric expenditure through a sequence of corrections. However, it has to be kept in mind that the following calculations can only be seen as approximations.

At 100% VO_2peak, cycling requires 35% of the maximum voluntary contraction force of the quadriceps[147], whereas in an isolation exercise such as knee extension exercise nearly all of the spent energy is used on the contraction and supporting processes in the muscle itself, getting close to 100% of its maximal contraction potential at 1RM. Assuming ATP requirements increases linearly with increasing contractile recruitment, comparing the two exercises, 2.9-times more cycling time should be done. Calculating with an intensity of 75% of VO_2peak, the mean of the range of typical endurance exercise of 70-80% and a level used for endurance exercise in the comparative **papers IV-V**, and a typical speed of 60 RPM would mean the quadriceps is recruited to 25% of its maximum force potential. Consequently, from the viewpoint of contractile work of the quadriceps, one would have to do 4-times the whole-body work in cycling compared to leg extension to achieve an energy expenditure-based similarity of both.

For an average male person (80 kg BW, 40 years) 30 minutes of cycling with an average heart rate of 160 bpm at 75% VO_2peak would consume 460 kcal (15.3 kcal/minute). In resistance exercise, knee extension exercise at 80% of 1RM (at a self-chosen speed with an average of 8 reps in 33 seconds; ~4 seconds/rep) consumes 25 kcal/minute in total[148]. With such a self-chosen exercise speed, one minute would correspond to 15 reps (in two separate sets), meaning 1 rep costs 1.67 kcal. Concentric exercise, depending on intensity level of the exercise, is between 2.5- and 6-times more energy consuming than eccentric exercise[149–151], in average intensity exercise it can be assumed as being 4-times more energy consuming[152]. It can be assumed that a typical, self-chosen speed that takes 4 seconds per rep would be 1.5-0-2.5-0 (seconds; concentric-break-eccentric-break). Therefore, the concentric 1.5 seconds cost 1.18 kcal (0.79 kcal/s) and the eccentric 2.5 seconds of one rep cost 0.49 kcal (0.2 kcal/s). On a side note, it is also worth mentioning that 40% of these energy costs are actually non-contractile[153,154], but rather invested in other related energy-consuming processes such as maintaining the activity of ion pumps.

As concluded before, to achieve an energy expenditure-based similarity of both forms of exercise, cycling should be 4-times the work of the work performed in leg extension exercise. So, corresponding to the 460 kcal consumed in 30 minutes of cycling endurance exercise, a matching resistance exercise should therefore consume 460 × 0.25 kcal - 115 kcal. As one rep at a self-chosen speed (typically and here: 1.5-0-2.5-0) consumes 1.67 kcal, this would correspond to roughly 68 reps. If choosing the same (self-chosen, to-exhaustion at 80% 1RM) set length as Reis et al. demonstrated to result in 25 kcal/min with 8 reps, this would result in 8.6 sets at 8 reps[148]. To account for a possible faster execution, the number of reps can be rounded up to 9 sets at 8 reps. However, it has to be kept in mind that such a correlation can only hold true for the given exercise duration of 1.5-0-2.5-0, which would be a typically execution speed seen in leg extension.

So, to conclude based on energy expenditure:

<u>30 mins @75% VO_2peak</u> matches to <u>9x8 repetitions @80% 1RM</u>

Some factors that can influence the previous calculation should be kept in mind when analyzing lower limb skeletal muscle which is usually sampled from *M. vastus lateralis*. Generally speaking, open chain exercises such as leg extension have a more pronounced effect on *M. rectus femoris*, while closed chain exercise such as leg press or squats recruit more of *M. vastus lateralis* and *medialis*[155], which should be considered when designing experiments. Also, the range of motion (ROM) of the joint mainly affected by *M. quadriceps femoris*, the knee joint (*Art. genus*) has to be kept in mind. Anatomically, its ROM is 135°, but it is functionally limited to 117° and typically used to 60° only in walking. Because of occurrent shear forces in the joint itself, a limitation to 90° ROM in resistance exercise might be advisable. The power optimum in leg extension would be an angular speed of 110°/s, resulting in an optimal concentric movement duration of 0.8 seconds[156]. Furthermore, toe direction influences activation of different parts of the quadriceps in different areas of the ROM, with vastus lateralis activation being highest with a toe-in orientation at the lower end of ROM, changing to a slightly superior neutral position at the top of ROM[157]. In summary, when optimizing an experimental design, it should be recommended to consider the implications of choice of specific exercise, range and angular speed of motion and toe orientation.

3.3 TISSUE AND DATA COLLECTION

3.3.1 Muscle tissue

For the studies that **papers I-V** are based on, skeletal muscle was collected from *M. vastus lateralis*. In the acute study of **paper I**, skeletal muscle was collected at five timepoints around the acute cycling intervention (Fig. 6A). In the long-term training study skeletal muscle was collected before and after the training period following a wash-out of at least 3 days (Fig. 6B). In the long-term study of **paper II**, skeletal muscle was collected before and one hour following an acute training session from both legs (Fig. 7). In the cross-sectional study **paper III** is based on, skeletal muscle was collected after a three day period without physical exercise (Fig. 8A). For **papers IV-V**, skeletal muscle was collected at four timepoints around the acute resistance or endurance exercise intervention (Fig. 8B). In all studies, muscle biopsies were collected under local anaesthesia using Carbocaine, in **papers I, III-V** by the Bergström needle technique under a vacuum[158] and in **paper II** using a Weil-Blakesley conchotome, snap frozen in liquid nitrogen using 2-Methylbutane as a secondary coolant and stored at -80°C. For **paper II**, the samples were then freeze-dried, powdered and dissected free of blood, fat and connective tissue. Collecting skeletal muscle using the Bergström needle technique is more invasive but results in greater yield, ranging from 200-400mg tissue, compared to the less invasive Weil-Blakesly conchotome that results in about 100mg of tissue.

3.3.2 Blood sampling

For the study that **paper V** is based on blood was sampled from *V. mediana cubiti* using a Cell Preparation tube (CPT; BD Vacutainer #362780) at the same four same timepoints around the acute intervention as the muscle biopsies. CPT tubes allow for the collection of peripheral blood mononuclear cells (PBMCs) which are comprised of lymphocytes, monocytes and dendritic cells[159]. Such cells can be isolated by density centrifugation with the Isopaque-Ficoll gradient technique[160]. Previous results

showed that ready-to-use PBMC systems such as CPT result in ~70% higher yield of cells from whole blood and also simplify and standardize sampling and isolation and can therefore be seen as practically superior to the classical Ficoll approach[161]. Using the CPT method, PBMCs were extracted after 30 minutes of centrifugation at 1700g. The plasma-PBMC mix was diluted to 50ml with 5% FBS in PBS and centrifuged for 15 minutes at 300g. Supernatant was decanted and the pellet resuspended in residual wash buffer by gentle tapping. PBMCs were washed again with 5% FBS in PBS and centrifuged for 10 minutes at 300g. The resuspended pellet was filled up with 3ml of 10% DMSO in FBS, frozen at a rate of -1°C·min^{-1} in three aliquots using the CoolCell LX cell freezing container and stored at -180°C.

3.4 MOLECULAR ANALYSES

3.4.1 Baseline analysis methods

To characterize the subjects in **papers III-IV**, enzymatic activity of citrate synthase was measured using a biochemical assay as previously described[162]. Citrate synthase is an enzyme of the citrate cycle that can be used as representation of mitochondrial function, is increased with regular endurance training and can be used to identify endurance athletes. In **paper III**, skeletal muscle fibertyping was performed using the myosin heavy chain (MHC) gel method which enables differentiation between type I, and type IIa and IIx as previously described[163]. In **paper IV**, fibertyping was performed by immunohistochemical methods, differentiating between type I and type II. For details about the antibodies used see **papers III and IV**. This method was also used to measure muscle fiber cross-section area (CSA) which allows for differentiation between highly hypertrophic strength athletes and untrained subjects.

3.4.2 RNA extraction and qPCR

For the studies that **papers I-V** are based on, RNA from skeletal muscle was extracted using a metal bead mill homogenizer and the phenol-based TRIzol (Invitrogen #15596-018) method. RNA was quantified spectrophotometrically and using a Bioanalyzer and equal amounts subjected to reverse transcription using Superscript Reverse Transcriptase (Fisher Scientific, #4368814). For qPCR in **paper I**, the TaqMan system was used (TaqMan Fast Universal PCR Master Mix, Applied Biosystems #4352043), for **papers II-IV** the SYBR Green system was used (SsoAdvanced Universal SYBR Green Supermix, Bio-Rad #1725272). qPCR was performed with a Bio-Rad C1000 touch thermal cycler. For specific primers see the respective paper.

3.4.3 Western blot and methylation analysis

In **paper I**, in addition to qPCR the investigated STARS pathway was also analysed on protein level by targeted western blot. Protein was extracted using a glass homogenizer and RIPA-buffer with a protease inhibitor cocktail (Roche Diagnostics) and then centrifuged at 15000g for 10 minutes. Gel separation was performed with 18µg protein using Tris-Glycine PROTEAN gels (Bio-Rad) and blotted on nitrocellulose membrane using the Trans-Blot Turbo system (Bio-Rad). Fluorescent secondary

antibodies were used for detection (Li-Cor IRDye and Odyssey system). For details about used antibodies see **paper I**. For **paper II**, DNA was extracted using the Gentra Puregene DNA kit (Qiagen, #158922) and bisulfite transformed using the EpiTect Fast Bisulfite kite (Qiagen, #59824). 10ng transformed DNA was used to perform targeted methylation analysis of the promoter region of the PGC-1α gene using the PyroMark PCR kit (Qiagen, #978703), the PyroMark Q96 ID device (Qiagen) and PyroMark Gold Q96 reagents (Qiagen, #972804). For details see **paper II**.

3.4.4 RNA sequencing

For **papers III-IV**, RNA sequencing was performed using ~2500µg of extracted RNA at the National Genomics Infrastructure (NGI) at SciLife Stockholm. Strand-specific Tru-Seq RNA libraries were prepared using poly-A selection. In **paper III**, sequencing was performed paired end 2x50bp in one lane of an S2 flow cell, in **paper IV-V** 2x150bp in two lanes using of an S4 flow cell with the Illumina NovaSeq 6000 sequencer resulting in an average sequencing depth of about 28M reads. Reads were processed using Kallisto[164] (**paper III**) the STAR aligner (**paper IV-V**).

3.4.5 Immune Cell Analysis

For **paper V**, cryo-conserved PBMC cell stocks were subjected to flow cytometrical analysis using the BD FACSCanto II. Cell stocks were rapidly thawed, quantified and about 250000 cells used for immunostaining in two panels that covered nine different immune cell types. Cell reads were processed using the FlowJo software. For specific antibodies and the gating strategy used for each panel see **paper V**.

3.5 STATISTICS AND BIOINFORMATICS

Following the collection and processing of the biological material and information, data was analyzed with different approaches based on their complexity. In **paper I-II**, statistics were calculated using SPSS v23 Statistica v13.3 and GraphPad Prism v7.03, in **paper III-V** using R v3.6 and RStudio v1.2. Across all studies, statistical significance was sent to $p<0.05$ and data was presented as mean ±SEM.

In **paper I**, statistics were calculated using two-way ANOVA with Dunnett's and Fisher's LSD post-hoc tests, baseline differences in subject characteristics were calculated using students t-test. Correlation analysis was calculated using two-tailed Pearson correlation. In **paper II**, ANOVA was used together with Fishers LSD and Bonferroni's post-hoc-tests. In **paper III**, results from Kallisto were used for calculation of differential gene expression analysis using DESeq2[165]. The results where then used for functional analysis using PIANO[104] and for gene ontology analysis using REVIGO[166]. Differentially expressed genes were then visualized using UpSet[167]. Based on differential gene expression data, GEMs were calculated using the generic human model (HMR) v2.0[109]. RNA sequencing data was also compared to publicly available type 2 diabetes and metabolic syndrome microarray datasets using limma[168] which were processed with the Jetset package[169]. For **paper IV**, differential gene expression was calculated using the edgeR package[170]. Repeated testing corrections were performed using the Benjamini-Hochberg method[171]. Differential gene expression results were then used to perform

log(FC)-based gene set enrichment analysis (GSEA) using the fGSEA package and functional annotations from the gene ontology database. Genome-scale metabolic modelling (GEM) was performed based on differential gene expression results using generic human model (HMR) version 2.00[109]. Motif activity response analysis (MARA)[114] was performed based on the FANTOM5 database of regulatory annotation[111,113]. Gene-motif correlations were performed using Pearson's product moment correlation coefficient. Gene-gene interaction networks were analyzed using the tissue and cancer-specific biological networks (TCSBN) database[172]. In **paper V**, baseline analyses were performed using ANOVA and t-test and immune cell population analysis was performed using ANOVA only. GSEA analysis was performed as in **paper IV**.

4 RESULTS AND DISCUSSION

4.1 EXERCISE-INDUCED EXPRESSION OF STARS

Exercise training and adaptation requires the transduction of an exercise stimulus and its translation into a signal of biological nature. **Paper I** aimed at investigating one such potential mechanisms, the STARS pathway of mechano-transduction of such a stimulus. Only a handful of studies have investigated the STARS pathway to date, however most of them focusing on individual elements such as STARS or MRTF-A, or gene expression only. Here we aimed at investigating a more complete STARS network including upstream effectors PGC-1α and ERRα on both gene and protein expression levels, introducing a single-legged long-term training design to enable an investigation of a potential systemic effect of exercise on the STARS pathway.

4.1.1 STARS pathway response to acute exercise

Thirty minutes and 2 hours after acute cycling exercise, STARS gene expression was upregulated 3.9-fold and 3-fold, respectively. Subsequently, levels gradually decreased towards baseline at the 24 hours timepoint (Fig. 9A). In response to acute exercise, gene expression of downstream targets of the STARS pathway were stimulated in a similar way but to a lesser extent (Fig. 9B,C). Even though STARS protein visually displayed an increasing tendency over the 24 hours following acute exercise, no statistically significant changes could be observed (Fig. 10A). This finding is likely due to a large variation of protein expression between subjects, which implies that more subjects should have been included. Interestingly, considering that the mechanism of MRTF-A is directly affected by the presence of the STARS protein, the increase in MRTF-A mRNA would have to be explained by an alternative mechanism of actin polymerization (Fig. 3) by for example RhoA as suggested previously[25]. The here observed effect, increased SRF and MRTF-A gene expression without the presence of increased STARS protein has been reported previously[38,54]. However, as the proposed effect of STARS ultimately results in the translocation of previously G-Actin bound MRTF-A protein into the nucleus, changes in protein levels of SRF might not be required for

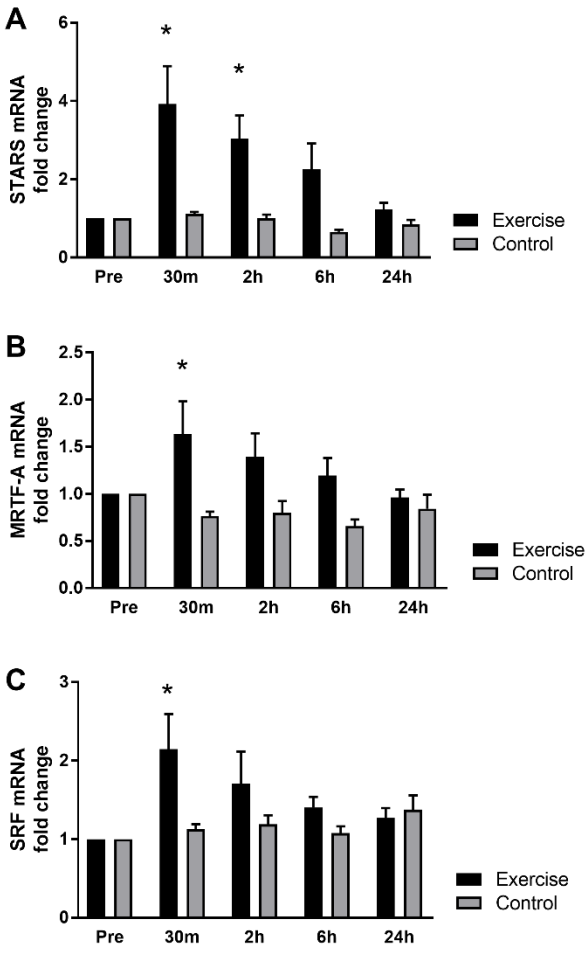

*Fig. 9: Effect of acute exercise on STARS (**A**), MRTF-A (**B**) and SRF (**C**) gene expression. (*: p<0.05 compared to pre)*

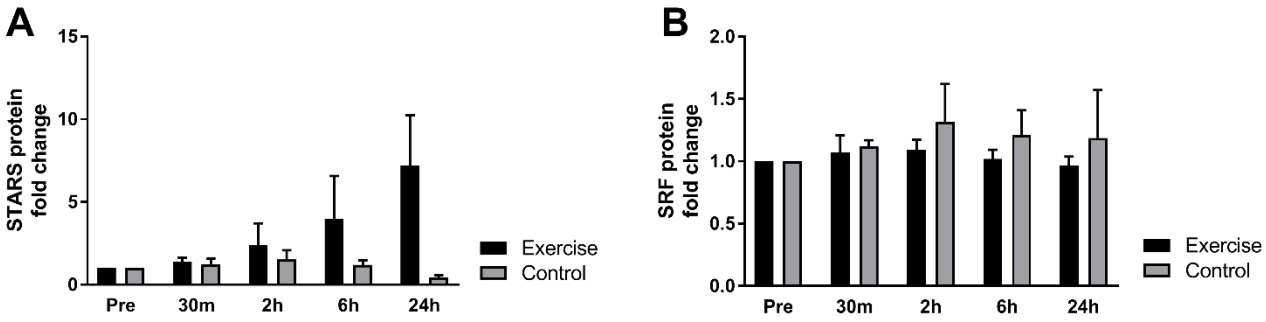

*Fig. 10: Effect of acute exercise on STARs (**A**) and SRF (**B**) protein expression.*

increased SRF transcriptional activity as such an increase is triggered simply by the change of the sub-cellular location of the MRTF-A protein. While not measured directly in the present study, acute exercise is widely known to activate transcription of SRF downstream targets which can be assumed to happen during the acute intervention[27,28,173]. In summary, acute exercise stimulates gene expression of the STARS downstream axis but apart from the non-significant increase of STARS protein over time has no effect on downstream protein expression which could be explained by the transcriptional activation being based on sub-cellular localization rather than numeric increase. However, while the here presented study added some knowledge to the acute regulation of STARS, further insights into other actors of the STARS network (see Fig. 3), for example RhoA would be required to more thoroughly uncover the dynamics of the STARS pathway. Furthermore, to explain the response of the central exercise effector SRF[174], an in-depth analysis and inventory of its upstream regulators would certainly be of great benefit.

4.1.2 Long-term training effects

Following a 12-week one-legged knee extension endurance training period, there was no change in baseline protein levels in any of the here investigated elements of the STARS pathway. STARS mRNA was not significantly different after 12 weeks of training compared with the pre timepoint or with the untrained leg (Fig. 11C). At the mRNA level, PGC-1α, an upstream effector of STARS, was significantly upregulated in the trained but not in the control leg (Fig. 11A). Also, ERRα mRNA showed a tendency to increase with previous training (Fig. 11B). Gene expression of the downstream factor MRTF-A was significantly increased in the trained leg (Fig. 11D). Both, PGC-1α and ERRα are prominent exercise-related genes that have repeatedly been shown to be extensively influenced by acute exercise[39,49,51,52,175,176]. These seem to herein be more profoundly stimulated than STARS, however, not surprisingly given the central role of PGC-1α and ERRα for pathways other than STARS related to exercise. In both the acute and long-term part of the study we show that 24 hours following an acute bout of exercise STARS is not significantly upregulated. As previously discussed, MTFT-A is associated and bound by monomeric G-actin. An increased amount of actin following 12 weeks of training and a certain amount of hypertrophy could explain the increased MRTF-A gene expression at resting baseline. However, over-all, the results do not allow for a conclusive evaluation of such an isolated view at the STARS pathway. Previous studies investigating the STARS pathway after an extended period of training saw increases in STARS gene expression, however following a resistance training

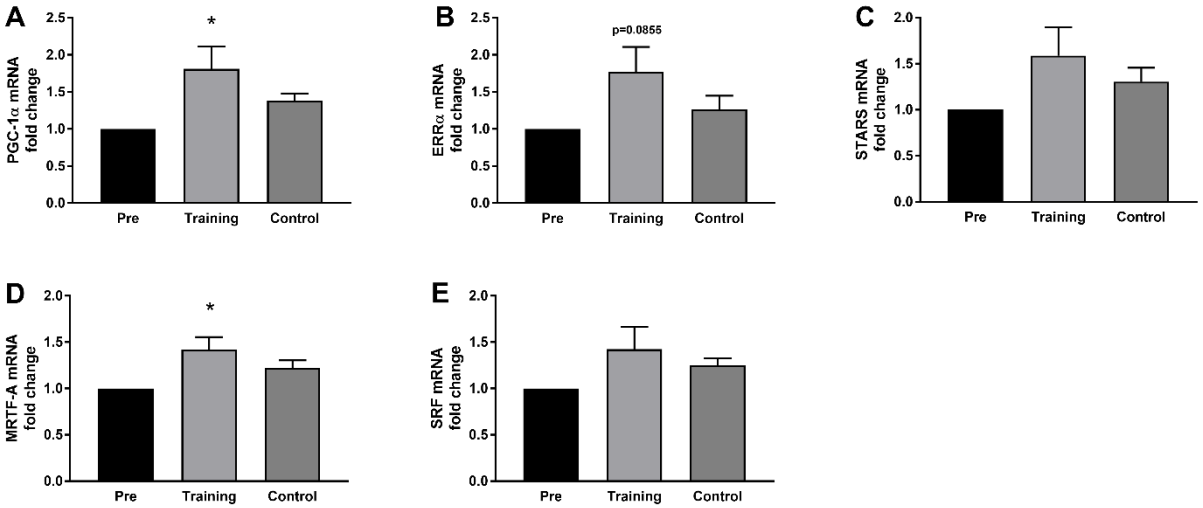

Fig. 11: Gene expression of members of the STARS pathway before and following a 12-week one-legged knee extension training. (: p<0.05 compared to pre)*

protocol or exhaustive cycling[53,54], both resulting in high local exercise intensity that might be required to induce an increase in mRNA expression. Furthermore, considering the complex interdependent regulatory network of signaling in the context of exercise, while STARS might contribute to the exercise response, it might only play a minor role that together with other small changes result in larger over-all effects. Small shifts such as these observed with STARS might be an effect that, taken together with other small shifts, could make a difference in the context of exercise but certainly are harder to isolate and elucidate.

4.2 A MECHANISM FOR MUSCLE MEMORY

While it has previously been observed that a history of exercise training and adaptation can positively influence the speed of recovery from an episode of disuse, the mechanism behind such observations is not entirely understood. To improve our knowledge about the molecular background of faster recovery, **paper II** aimed at investigating key exercise-related gene and protein expression and epigenetic and protein modification in a study design based on unilaterally pre-trained muscles.

4.2.1 Previous training affects acute exercise gene expression

19 volunteers (10 males, 9 females) were subjected to 10 weeks of unilateral leg resistance training (3x/week leg press and leg extension exercise with resistance based on 1RM-re-tests, for details see **paper II**) and 20 weeks of detraining, after which they showed a significantly higher strength in the previously trained leg (see **Paper II)**. Gene expression was analyzed in skeletal muscle samples taken before and 1h after an acute bout of unilateral leg resistance exercise (alternating the exercise leg with each set) following this training/detraining period, with the selection of target genes based on previously reported epigenetic hypomethylation of a range of target genes in a comparable training/detraining protocol[10]. Differences between the legs were also found in gene expression of total PGC-1α, PGC-1α exon 1a, and a number of other exercise-related genes (see **Paper II**, Figure 3), including the novel exercise gene SPRYD7 that is enriched in skeletal muscle and has been previously linked to body mass[177,178]. Interestingly, resting baseline mRNA levels of SPRYD7 as well as PGC-1α

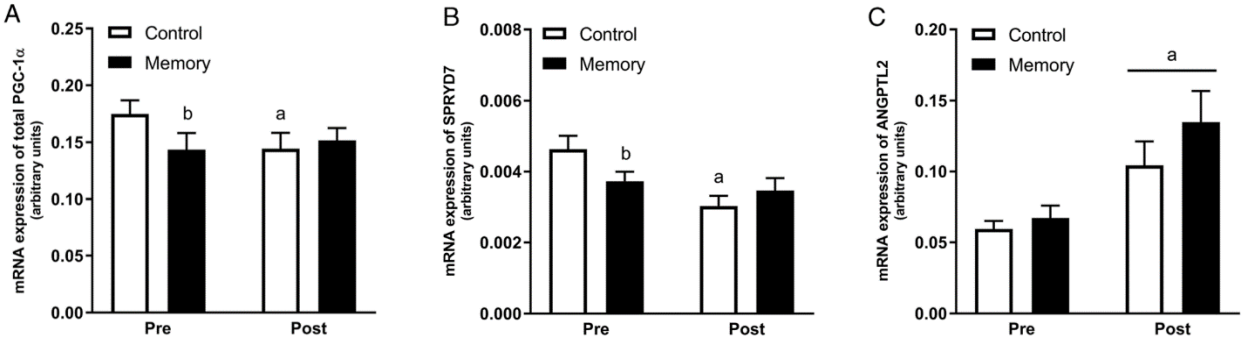

Fig. 12: A previous period of unilateral training (Memory leg) influences gene expression both at baseline (pre) but also after a unilateral leg training session in both legs (post) in PGC-1α (**A**)*, SPRYD7* (**B**) *and ANGPTL2* (**C**)*. (a: p<0.05 compared to pre timepoint of the same leg; b: compared to the other leg at the same timepoint; horizontal line represents a main effect in the analysis of variance)*

were lower in the previously trained leg (Memory leg) than in the previously untrained leg (Fig. 12A,B). The lower expression in the Memory leg at baseline was accompanied with no significantly different level of mRNA expression following an acute bout of exercise, while in Control leg the gene expression decreased. In another gene, ANGPTL2, previous training resulted in on average about 25% expression following acute exercise compared to Control leg (Fig. 12C). Other genes analyzed in **paper II** that previously have been reported to be hypomethylated and attenuated in expression in response to previous training[10] showed no memory effect (AXIN1, TRAF1, UBR5) or rather a suppressing memory effect (SETD3, FBXO32, TRIM63). The differences with previous, unilateral training and detraining on the gene expression levels of exercise-relevant genes (for further results see **paper II**) together with increased strength in the Memory leg physiological testing could suggest the existence of a memory effect. Possible mechanisms for such differences in gene expression and a memory effect could, amongst others be epigenetic modification by hypomethylation of regulatory motifs of such genes or the phosphorylation of proteins that play a role in transcriptional regulation or protein synthesis. However, when separating the results from the men and the women, the majority of the differences in gene expression can be explained by variations seen in the men with only a few exceptions, while in women changes were largely insignificant (see **paper II**, figure 2, 3). In previous papers from our lab have shown that PGC-1α gene expression is elevated 2 hours after acute exercise[41,179], which is a later timepoint than the 1-hour timepoint used in **paper II**, which could partially help to explain the decrease or non-regulation shown herein. However, examination of one PGC-1α variant, the PGC-1α exon 1a, shows a 50% increase of expression following acute exercise in the Memory leg only (**paper II**, figure 2C). As we showed in a previous publication, several different splice variants of PGC-1α exist and are regulated to a different extent by exercise[41] which could further explain the discrepancy between the response of PGC-1α and its exon 1a isoform in **paper II**. The differences in gene expression between the legs despite the same relative exercise load would therefore likely have to be explained by other mechanisms, suggesting the existence of a "memory effect" on the transcriptional level that can manifest either in reduction or augmentation of gene expression and could be based on epigenetic or protein modification as mentioned above.

4.2.2 DNA methylation and protein phosphorylation

To investigate mechanisms that could explain the observed differences in PGC-1α gene expression, targeted epigenetic modification analysis in the proximal promotor region located in exon 1a was performed at 7 of 49 CpG sites. No differences were observed in methylation status in the 7 sites. The possibility for an epigenetic explanation for the observed memory effect cannot be excluded since histone modifications or DNA methylation alterations in the remaining 42 CpG sites could be present. Another mechanism that could exert some form of memory effect on the response to acute exercise could be the exercise-induced rate of protein synthesis. It has previously been reported that a history of strength training increases the basal protein synthesis rate but reduces the synthesis rate increase with acute exercise[67–69], and that such adaptations are reduced with detraining[180]. Conflicting data exists regarding mTOR-related signaling[57,67]. Nevertheless, mTOR has previously been reported to be affected by chronic strength training[57], indicating a possible muscle memory. In the Memory leg only, phosphorylation of the mTOR-target protein 4E-BP1 (Fig. 13A), which has an important role in protein synthesis was increased at its ^{Thr46} and ^{Ser65} locations. Acute exercise resulted in a change of phosphorylation in the proteins eEF2 (decrease; Fig. 13B) and AMPK (increase; Fig. 13C), which have roles similar to 4E-BP1. Interestingly, the control leg compared to the Memory leg at location ^{Thr56} and ^{Thr172} had around 18% and 14% lower phosphorylation respectively. Other elements of the protein synthesis machinery that are upstream of the previously mentioned proteins such as mTOR, S6K1 and S6, while significantly increasing with acute exercise showed no difference between the two legs. In summary, while central upstream mechanisms of protein synthesis such as mTOR do not seem to be affected in their phosphorylation status by previous training, downstream elements of this machinery which could be responsible for fine tuning of the protein synthesis process did exhibit a memory effect, specifically in increased phosphorylation of 4E-BP1 in the Memory leg following acute exercise. While such a modification difference can have an effect on over-all protein synthesis, its origin is unclear. However, one possible explanation of such a difference could be a change in phosphatase activity[181] with previous training, a mechanism that could be a potentially interesting subject of future research of muscle memory effects.

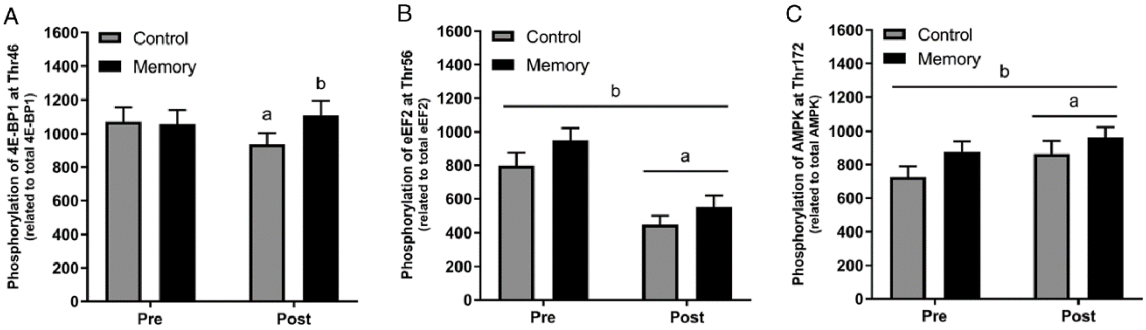

Fig. 13: A previous period of unilateral training (Memory leg) influences phosphorylation of proteins in the protein synthesis machinery: 4E-BP1 (**A**)*, eEF2* (**B**) *and AMPK* (**C**)*. (a: p<0.05 compared to pre timepoint of the same leg; b: compared to the other leg at the same timepoint; horizontal line represents a main effect in the analysis of variance)*

4.3 EFFECTS OF LIFE-LONG HIGH-LEVEL TRAINING ON PERFORMANCE AND PHYSIOLOGY

Both mechano-transduction and muscle memory over time can contribute to the large difference between high-level athletes and untrained individuals[6,70,81,90]. While the performance and health effects of such a difference is well established its molecular and mechanistic background is relatively unknown. To address this, **papers III-V** aimed at investigating the effect of life-long high-level training on the transcriptome (**paper III,IV**), metabolome (**paper IV**), regulome (**paper IV**) and the immune system (**paper V**).

4.3.1 Baseline differences following life-long training

Analysis of skeletal muscle biopsies from *M. vastus lateralis* of 40 subjects at rest showed background-specific differences on enzymatic, histological, transcriptomic and metabolomic levels. Citrate synthase (CS) activity of endurance athletes was about 80% higher in both male and female endurance athletes (ME, FE) compared to sedentary controls (MC, FC). Such a difference in CS activity has been reported before[71,182] and is linked to increased mitochondrial activity in skeletal muscle in response to endurance training. Furthermore, fiber composition was significantly different between groups, with both MC and FC having significantly higher proportions of type IIx fibers, while male and female endurance athletes (ME, FE) had significantly more type I fibers compared to MC and male strength athletes (MS) and FC, respectively. Such differences in fiber types are consistent with previous findings and credited to fiber type conversion from IIx to IIa with physical activity, and targeted hypertrophy of both type I and type IIa fibers in response to endurance or resistance training respectively[183–185]. Results from RNA sequencing revealed that on the transcriptomic level, the largest numbers of differentially expressed genes (DEGs) were found in the comparisons MEvsMC and FEvsFC with 1097 and 1711 DEGs, respectively. Furthermore, the numbers of DEGs were smaller comparing MEvsFE (135 DEGs) than in MCvsFC (452 DEGs; Fig. 14). The smallest differences existed in MSvsMC (26 DEGs). Results from previous publications support the herein reported shift of the transcriptome with life-long endurance training and it is likely that such a change is associated with a reprogramming of the transcriptome in favor of aerobic respiration capabilities, as also concluded in several publications[95,97,186,187]. As a main novel contribution from **paper III**, results confirm such a shift over a

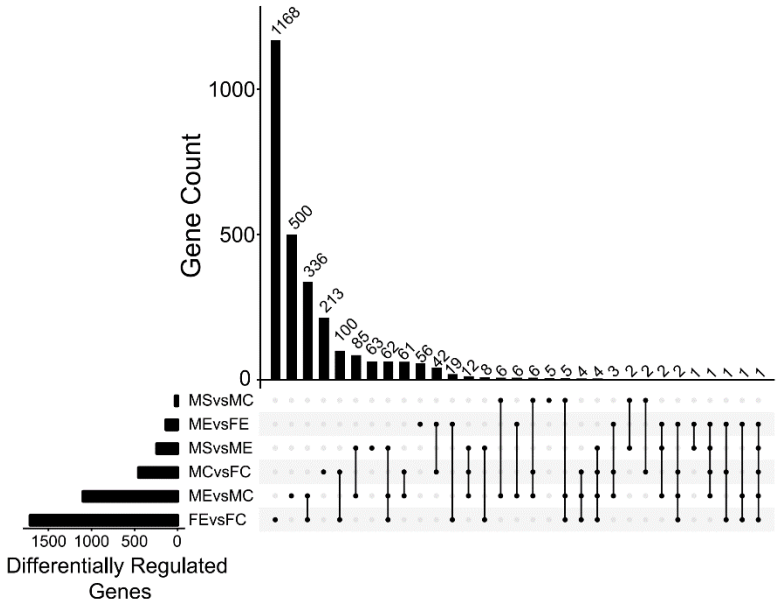

Fig. 14: Number of differentially expressed genes (DEGs; bottom left) and shared DEGs between comparisons (top right) of male and female control groups (MC, FC) to endurance (ME, FE) and strength trained athletes (MS).

life-long career of endurance training. Furthermore, similar differences between endurance trained and control subjects of the same sex reported in **paper III** were reported before[187]. About 50% of the genes found to be differentially regulated in MEvsMC were also found to be differentially regulated following a 3-month endurance training period in men. And about 30% of the differentially regulated genes in FEvsFC were also found to be differentially regulated following the same 3-month endurance training period in women. The previous notion of a shift in genes relevant for aerobic respiration is supported by a gene ontology analysis of DEGs in **paper III** which revealed that the differences between endurance athletes and sedentary controls was mostly associated with cellular respiration and the tricarboxylic acid (TCA) metabolism in both men and women (see **paper III** Figure 3A,B). The RNAseq-based differences in energy metabolism were phenotypically confirmed by enzymatic assay of citrate synthase which was significantly higher in endurance athletes compared to control subjects (**paper III**, Figure 1C). Regarding sex difference in sedentary controls, DEGs were mostly associated with protein catabolism which was elevated in men, however comparing ME and FE, gene ontology analysis showed higher activity of oxidative mitochondria-related and metabolic pathways (**paper III**, Figure 3C,D). The same differences were further confirmed by genome-scale metabolic modelling results, revealing amongst others BCAA catabolism, fatty acid oxidation and TCA cycle as largely affected by life-long high-level endurance training resulting in a higher activity in ME and FE compared to MC and FC. A closer investigation of the TCA cycle revealed that key metabolites such as Acetyl-CoA were significantly more abundant and enzymes such as CS were expressed significantly higher in response to life-long endurance training (Fig. 15). Taken together, the discovered differences of endurance trained athletes largely depending on energy metabolism and results from CS

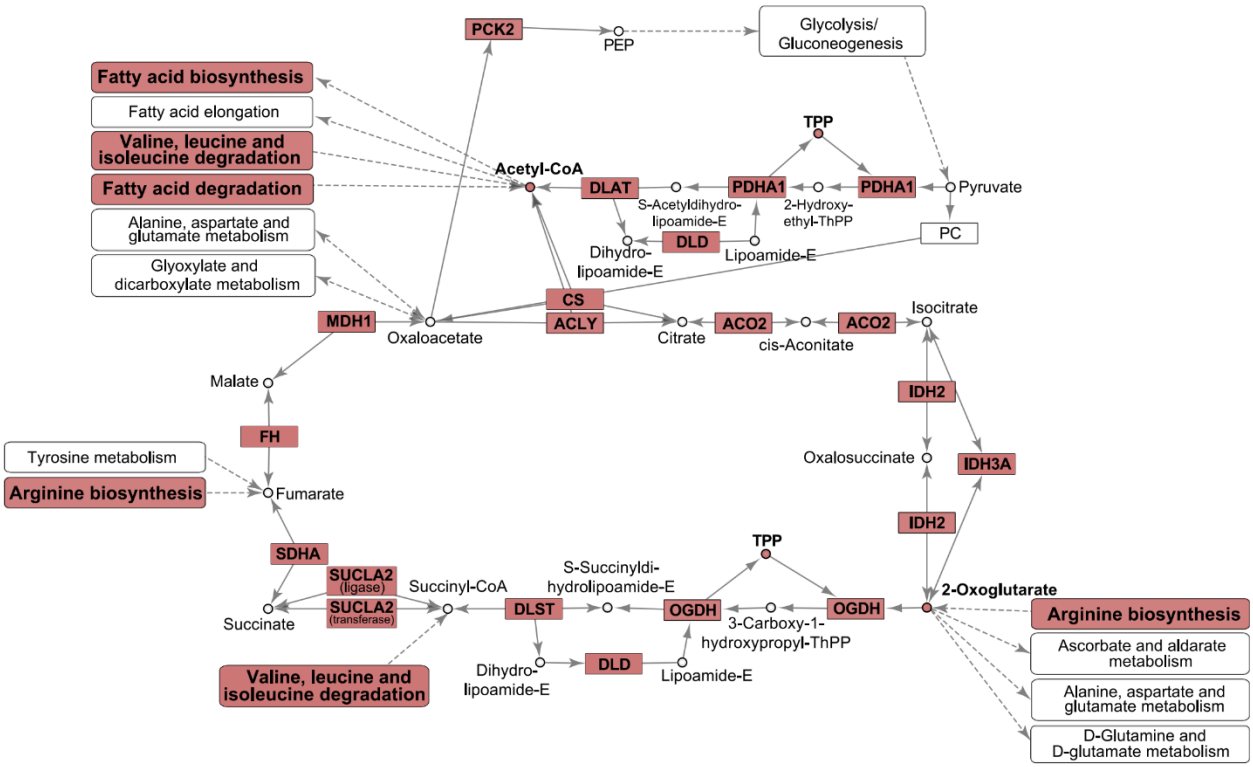

Fig. 15: Genome-Scale metabolic modelling of differences between male endurance athletes and sedentary controls in the TCA cycle. Red colored metabolites, enzymes and pathways are more active in endurance athletes.

activity assays suggest a major adaptation to be an increase in the capacity of the muscle for mitochondrial metabolic processes. The increased capacity, based on structural adaptations in the muscle cell could contribute to the resilience of individuals with extensive endurance exercise background to prolonged periods of detraining and contribute to a concept of muscle memory on a cellular rather than on a molecular level, potentially adding another dimension to the concept of "muscle memory" mentioned in **paper II**. While results largely show the difference of endurance athletes to untrained individuals on molecular level, strength athletes seem to be rather close to untrained individuals. However, in a similar way, the resilience of strength athletes to detraining could be based on a cellular memory process - the physical accumulation of muscle mass - rather than solely on molecular principles.

To put these adaptations into a health context, DEGs from the comparisons MEvsMC and FEvsFC were integrated with datasets from training intervention studies of males with type 2 diabetes (T2D) compared with subjects with normal glucose tolerance (NGT)[188] and females with metabolic syndrome (MetS)[189] compared to healthy subjects. Results show that following 1 year of training in T2D men, the number of DEGs regulated in the opposite direction decreased substantially while DEGs regulated in the same direction increased. Similarly, DEGs regulated in opposite direction in women with MetS decreased following 6 months of training. Specifically, oppositely regulated genes that reverted in response to a period of training were associated with pathways related to high blood glucose, and insulin resistance. These comparisons show that on the gene expression level, a period of exercise training can make metabolically unhealthy individuals more like endurance trained athletes which are metabolically healthier. Such a transformative process with exercise training over a 6- to 12-month period may also be viewed as a form of memory of (non-)exercise and diseases such as T2D and metabolic syndrome. Such diseases can result in self-reinforcing metabolic and systemic mechanisms. For example T2D can result in muscle loss[190], which in turn would reduce the size of the metabolic buffer that is skeletal muscle. Chronic hyperglycemia can negatively regulate aerobic adaptation, in turn inhibiting exercise-related metabolic improvements that could help manage hyperglycemia[191]. A self-reinforcing mechanism would as a result delay a reversion by exercise and create a "dragging" memory effect that can, in its basic principle be seen as in way similar to a memory effect to training, however in an antithetical sense.

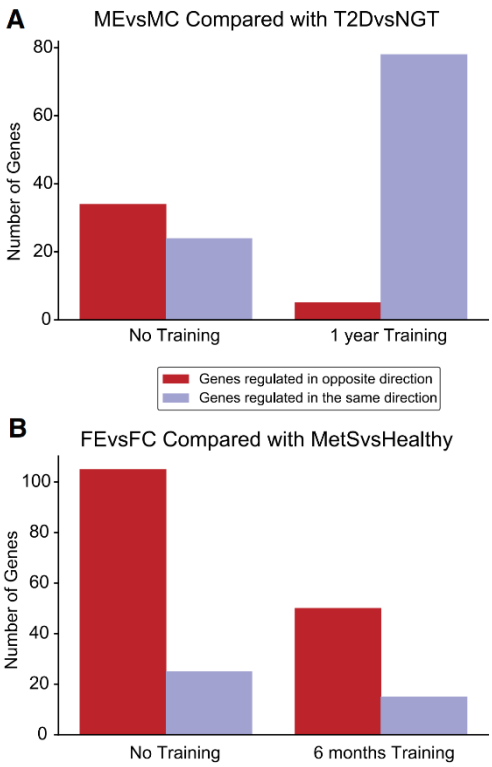

Fig. 16: Comparison of DEGs in ME vs MC and persons with type 2 diabetes (T2D) vs persons with normal glucose tolerance (NGT) before and after a year of training (**A**) *and FE vs FC and individuals with metabolic syndrome (MetS) vs healthy subjects* (**B**) *after 6 months of training.*

4.3.2 Life-long high-level training influences the acute exercise response

The analysis of skeletal muscle biopsies from 24 men before and after an acute bout of endurance (EE) or resistance exercise (RE) in **paper IV** confirmed the results from **paper III** regarding baseline differences. **Paper IV** added compelling new information about the influence of exercise history and type of acute exercise on transcriptomic, metabolomic and regulomic levels. At rest, the endurance group was largely different from both control and strength groups with 1363 and 919 DEGs, respectively. In contrast, comparing control and strength group only 57 genes were differentially expressed. Following acute exercise, the number of DEGs compared to the pre timepoint gradually increased following both types of exercise, however there was a larger response to RE (Fig. 17). Interestingly, the control group stands out in this comparison with its number of DEGs at 3h following EE almost being twice as high compared to the endurance and strength athlete groups. When interpreting such results, it has to be kept in mind that this analysis represents a local gene expression effect in *M. vastus lateralis*. While in resistance exercise most of the work is performed and its gene expression consequences therefore would rather be visible locally in the isolated muscle, endurance exercise involves a substantial amount of systemic work, involving increased blood flow, heart rate and energy mobilization, effects potentially not visible with such a local perspective on skeletal muscle[192–194]. Whereas both athlete groups are used to perform intense muscle work to some extent, sedentary controls are less used to such a high demand on the skeletal muscle which could result in a high level of local effort and stress which potentially can lead to a larger amount of DEGs[97,195]. Furthermore, it could be observed at the 3h timepoint, athlete groups had a higher number of DEGs in response to the exercise they were unfamiliar with compared to the group that was familiar with the exercise (strength group doing EE: 56% more than in endurance group; endurance group doing RE:

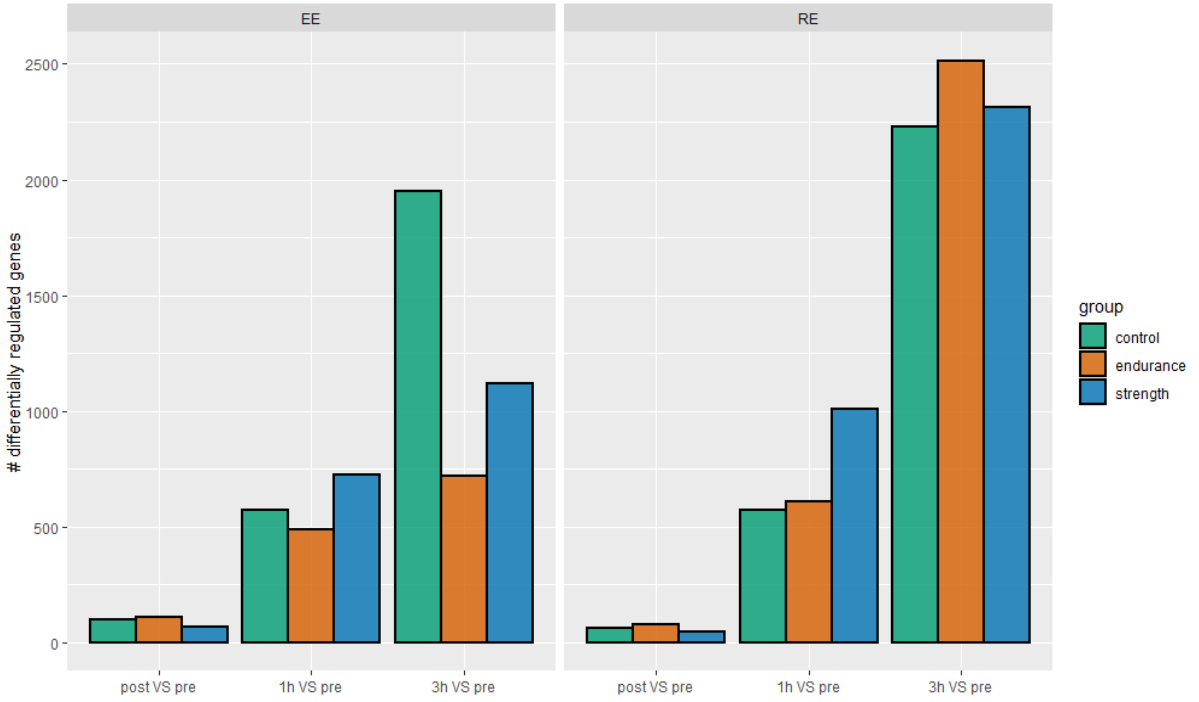

Fig. 17: Number of differentially regulated genes in the three subject groups (untrained control, endurance trained and strength trained) comparing three timepoints following acute endurance (EE) or resistance (RE) exercise - post (directly after exercise), 1 h after and 3 h after to before exercise (pre).

9% more than in strength group), a potential blunting effect that has previously been reported in connection with exercise that would be in line with the increased numbers of DEGs in control group in response to EE (Fig. 17)[52]. Furthermore, RE was generally shown to result in a higher number of DEGs that were shared between all three groups with early timepoints more homogenous across groups than later timepoints (**paper IV**, Fig.2B). Together, this could suggest on the one hand that RE depends on a higher number of "core" genes compared to EE. And on the other hand that a potential advantage of the previous training experience could rather lie in a difference during the progression into regeneration from acute exercise happening during later timepoints rather than in the early timepoint close to the acute exercise itself. Considering resistance training with short bouts of muscle contraction a potentially more local effort that largely depends on the skeletal muscle itself compared to a more complex systemic effort such as endurance exercise, the higher complexity and larger dependence on other organ systems in the more systemic effort of endurance exercise[192,194] could explain such a divergence. In a similar sense, increasing divergence of exercise responses over time comparing EE and RE could potentially be explained by an increasing systemic influence on the

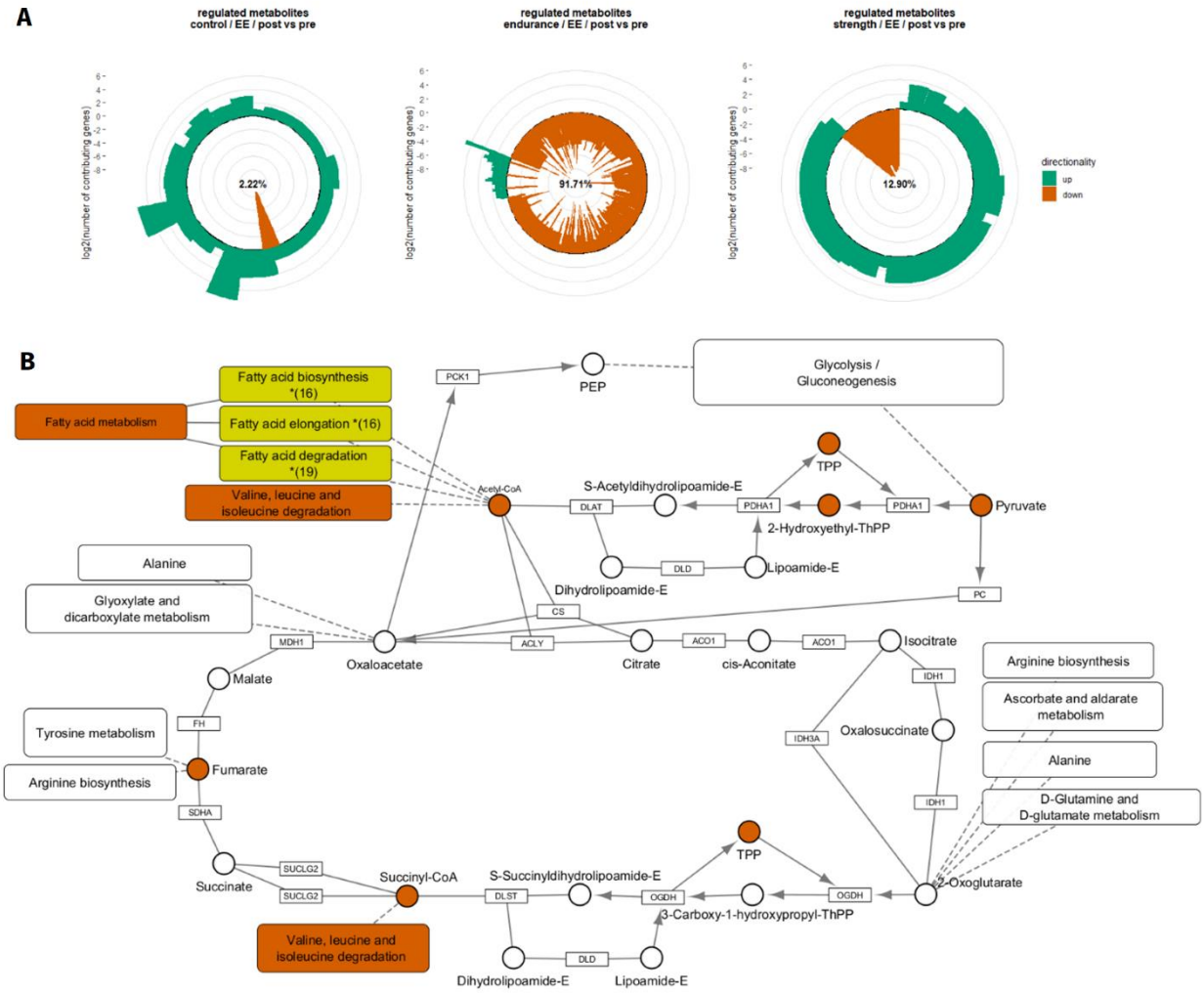

Fig. 18: The inferred metabolic response to acute endurance exercise at the timepoint immediately post. Directionality of regulated metabolites at timepoint post in response to EE in the three groups (**A**)*. Details of the regulated metabolites and pathways in the TCA cycle in endurance athletes at timepoint post in response to EE, orange represents downregulation, pathways in gold have large numbers (in parenthesis) of their member metabolites downregulated* (**B**)*.*

regeneration following an acute bout of exercise for example via the endocrine system or from circulating cytokines as recently shown in the expression of a number of metabolites and proteins[196,197].

Furthermore, **paper IV** analyzed metabolomic changes in response to acute exercise by using the bioinformatic GEM method to predict metabolite abundance and enzyme activity. Amongst other results we showed that EE largely leads to an upregulation of metabolites, while RE shows a mixed up- and downregulation (**paper IV**, Table S2A). Very interestingly, one exception was the endurance athlete group that responded with the downregulation of about 92% of all regulated metabolites in response to EE while both the other groups largely responded with upregulation (Fig. 18B). The downregulated metabolites were largely associated with amino acid metabolism and the TCA cycle by KEGG mapping (Fig. 18A, **paper IV**, Table S3). Interestingly, such a difference between groups already exists at baseline, where comparing the endurance group with both the other groups results in about 1000 metabolites being significantly higher expressed in endurance group but only small differences between control and strength group (**paper IV**, Table S2A). As demonstrated above, many of the metabolites are associated with energy producing processes. While interpretation of differences might be difficult due to the complexity of the metabolism, many of the molecular processes of the energy production are located in mitochondria, which are largely affected in their function and abundance by life-long endurance training and could therefore help explain such differences. However, such a seemingly large difference as a result of the life-long high-level adaptation of endurance athletes

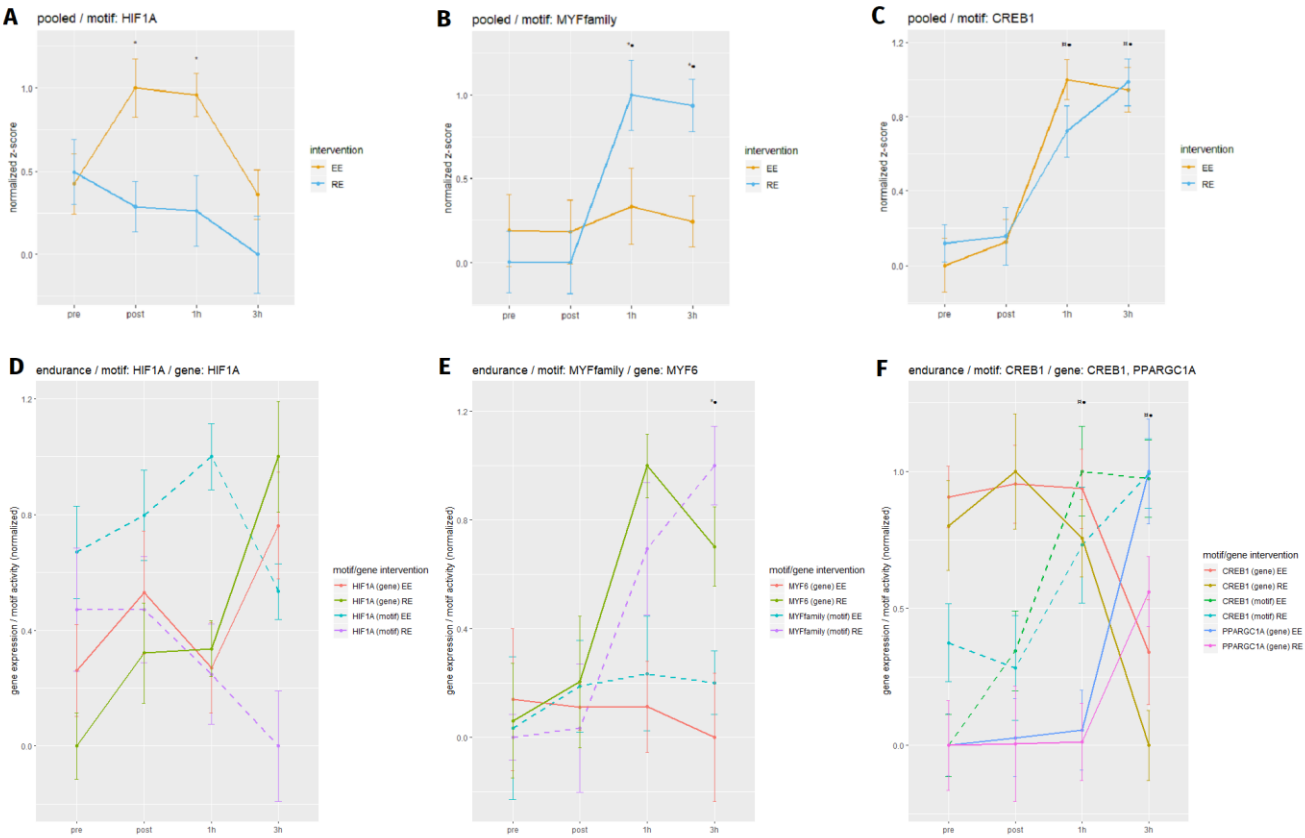

Fig. 19: Transcription factor motif analysis results of three motifs (**A-C**) *and their association with transcription factor coding and target genes with motifs pictured with dotted lines* (**D-F**).

on metabolic level adds another interesting layer of information and, after indications of the similarity of control and strength group were visible already on transcriptomic level, reinforces such a notion by contributing additional information about specific metabolic differences.

To add yet another layer of information, motif activity response analysis (MARA) was performed. While both the transcriptome and the metabolome peaked in their numbers of DEGs and differentially regulated metabolites at the 3h timepoint, the number of transcription factor (TF) motifs with significantly increased inferred activity peaked at the 1h timepoint. When investigating these motifs, some of them especially stand out regarding what kind of acute exercise they are stimulated by (**paper IV**, Fig.6A-C). Particularly, the HIF1A motif showed to be responsive to EE only, while the MYFfamily motif did respond to RE rather than EE (Fig. 19A,B). The activity of these motifs also showed to be affected by exercise background. The HIF1A motif activity was higher in endurance athletes compared to resistance athletes at the 3h timepoint, and MYFfamily motif activity was lower in endurance group compared to control group at 1 and 3h timepoints (**paper IV**, Table S4). Generally, the endurance group showed a largely lower motif activity compared to the control group. Taken together, this could suggest that a regulatory adaptation of life-long high-level endurance training lies in improved regulation of highly exercise-relevant transcription factors such as HIF1A, while in contrast other motifs are less active as a consequence of such an adaptation. HIF1A is a TF that is responsible for the regulation of genes responsible for erythropoiesis and angiogenesis, aimed at resolving local hypoxic conditions that might evolve from high-level endurance efforts[198] and its effect is based on complex degradation/suspension of degradation process involving the von Hippel-Lindau protein[199,200]. Continued activation of this mechanism over the career of a high-level endurance athlete might optimize such a process, resulting in the higher sensitivity of the HIF1A motif activity shown here. Since the MYFfamily motif is closely tied to myogenic regulatory factors, amongst others MYF6, an important regulator of hypertrophic processes[201,202], its high activity following resistance exercise is therefore not surprising. However, it is interesting to mention that this regulatory motif highly important for the hypertrophic response following resistance exercise is somewhat blunted in persons having performed life-long endurance exercise, possibly suggesting a desensitizing effect to resistance exercise. Furthermore, the CREB1 motif, regulator of the key exercise gene PPARGC1A was consistently upregulated in response to both EE and RE. Interestingly, when comparing the gene expression of transcription factors and their target genes to the time course of activity of the aforementioned motifs, both genes and motifs largely followed mechanistic expectations (Fig. 19D-F). While the complex regulatory background of the HIF1A TF as discussed in **paper IV** could explain the HIF1A TF/HIF1A gene dynamics, MYF6, based on a more direct regulatory principle closely followed the MYF family motif activity. And while the CREB1 motif activity increased together with the expression of its target gene PPARGC1A, expression of the TF coding gene CREB1 interestingly decreases in expression in what could be a negative product-feedback-loop.

Taken together, the layer of regulomics, molecular mechanisms that can regulate transcription, adds another level of information to the here performed analysis, reinforcing the large difference of endurance trained athletes to both, resistance trained athletes and control subjects. Such a metabolic

differentness of life-long trained endurance athletes is manifested on the metabolic level with the extensive immediate downregulation of metabolites in response to EE, and a blunted response to the regulation of hypertrophy. In reference to **paper II**, this again indicates some underlying mechanism based on life-long training background and could suggest that a potential muscle memory extends and manifests well into regulatory and metabolic levels.

4.3.3 Immune system response of long-term trained athletes

In **paper IV**, peripheral blood mononuclear cells (PBMCs) was collected and analyzed by FACS from the same subjects and at the same time points as skeletal muscle biopsies in **paper IV**. The results show that both acute endurance (END) and resistance (RES) training can modulate the immune system in a largely biphasic manner. Such a general pattern of regulation has previously been reported in circulating immune cells and are caused by a complex concert of mechanisms, including contributions from reservoir pools, leukocyte adhesion molecules and immune cell deformability[203–207]. The adaptive immune system in particular exhibited a biphasic response shape. Interestingly, only athletes showed a significant change in CD56+^{dim} NK-cells in response to acute exercise which was larger in amplitude in response to END in both endurance and strength athletes (Fig. 20A). The immediate increase in circulating NK-cells might be explained by the contribution of NK-cells from marginal pools all over the body to the circulating blood. The circulating immune cells could subsequently infiltrate target tissues that have an increased demand for immune cell activity, resulting in a decrease in immune cell populations at the 1h timepoint. A similar response, however, to a much lower extent, was observed in the circulating population of CD8+ T-cells, which would have the same origin and destination as the NK-cells (Fig. 20B).

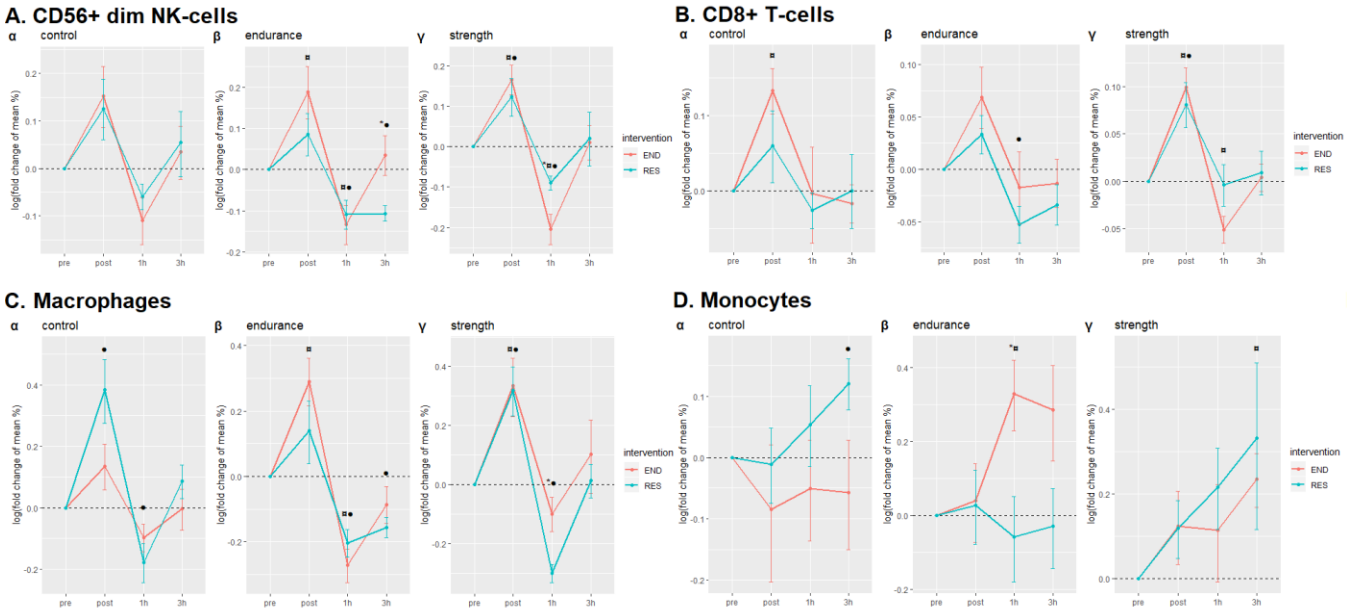

*Fig. 20: Circulating immune cell population response to acute endurance (END) and resistance (RES) exercise. (curves show fold change of mean % ±SE based on parent population (**paper V**, Fig. 1); adjusted p<0.05 by t-test; #: control vs endurance; †: control vs strength; *: END vs RES; ¤: timepoint vs pre (END); ●: timepoint vs pre (RES))*

The macrophage population, being part of the innate immune system also responded in a biphasic manner increasing its numbers in circulating blood by up to 40% in response to RES in control subjects (Fig. 20C). While endurance athletes respond significantly stronger to END, control subjects and strength athletes rather responded to RES. Macrophages originate from both monocytes infiltrating peripheral target tissue and locally maintained isolated monocyte populations by activation, however, monocytes residing in the lining of blood vessels can also be activated to macrophages, where they exert their function within the blood tissue[208,209]. The origin of macrophages from the circulatory system with its marginal pool could explain their biphasic response based on the same mechanism as mentioned above. Subsequently, macrophages play a major role for the regeneration of skeletal muscle in response to acute exercise[210] and have been shown to contribute to the regulation of inflammation but also myogenesis and vascularization processes[134,211]. Such a regenerative potential could explain the influence of exercise background on their abundance, as higher local muscle damage requires higher regenerative macrophage activity. Long-term endurance trained athletes, being more experienced in how to utilize their muscle to the functional limit in endurance efforts could exhibit more local muscle damage in response to END, while control and strength trained subjects reach higher levels of local damage in response to RES, requiring higher levels of macrophage activation and mobilization. In contrast to macrophages, monocyte mobilization results in a more diverse, and somewhat delayed response to acute exercise compared to other immune cell types (Fig. 20D). In control subjects, only RES resulted in a significant increase of circulating monocytes, whereas strength trained subjects responded to END only. Interestingly, a clear difference between exercise modalities was observed in endurance athletes, where END only resulted in up to 30% more circulating monocytes while RES had no effect. Monocytes are precursor cells to a range of immune cells, they originate from the bone marrow and exist in a permanently circulating but also locally residing pool[212]. This might help explain why immediate replenishment of such reservoirs from bone marrow might not be required resulting in the previously mentioned delayed, but still significant increase of their circulating levels. While some of these changes could potentially be explained by a

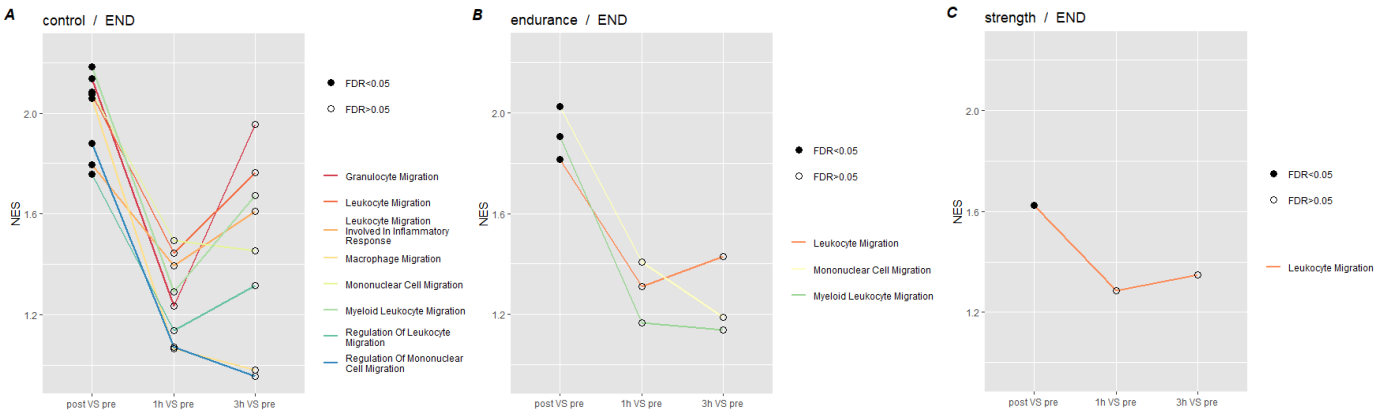

Fig. 21: Gene set enrichment analysis using the GO biological process database limited to immune cell migration terms. Skeletal muscle differentially regulated genes at the three timepoints following acute endurance (END, **A-C**) *exercise compared to the pre timepoint in the three subject groups control* (**A**)*, endurance* (**B**)*, strength* (**C**)*. Solid circles represent timepoints at which this term is significantly enriched, only terms with at least one significant timepoint are shown. (curve shows normalized enrichment score (NES) of each GO-term compared to the pre timepoint; FDR<0.05)*

previously reported immediate post-exercise shift in plasma volume[75], the disparity of the directionality in for example CD56+ dim NK-cells (Fig. 20A) and CD4+ T-cells (**paper V**, Fig.3D) would still suggest some influence of acute exercise on immune cell numbers. Furthermore, considering the increased plasma volume and the associated dilution of cells would rather result in a decrease than an increase in cell numbers. However, comparing the two populations mentioned above, the immediate increasing effect in NK-cells extends up to 20% while the immediate decreasing effect in CD4+ T-cells extends down to -10%.

Circulating immune cells are an integral part of the body's immune system at large. Skeletal muscle is integrated into the immune system as it is not only a target organ but also an endocrine organ expressing myokines which can influence other parts of the immune system in a crosstalk process[213,214]. However, skeletal muscle as a target organ can also be infiltrated by immune cells, particularly during high muscle activity such as exercise. Even though the present results show that an increased number of immune cells in blood, which at least indicates increased supply to the muscle, this is no direct proof of immune cell infiltration. To investigate a potential immune cell migration into and within the muscle, gene set enrichment analysis based on GO-terms of immune cell mobility was performed, investigating groups of genes functionally associated with immune cell mobility in skeletal muscle. Results showed, that genes responsible for immune cell mobility, particularly but not exclusively of the innate immune system were significantly enriched in response to END (Fig. 21A-C) and RES (**paper V**, Fig.4D-F). However, in response to both forms of acute exercise, untrained control subjects exhibited the most profound significant enrichment of terms associated with immune cell mobility (Fig. 21A, **paper V**, Fig.4D). Interestingly, in response to END, the response was highly coordinated and focused on the timepoint immediately following acute exercise. While this could potentially mean an increased infiltration of immune cells, coordinated with the increased supply of immune cells to skeletal muscle by circulating blood, another explanation for the increased mobility of immune cells might be that local resident immune cells increase their mobility in response to acute exercise and the demand for their regeneration potential. As previously discussed, a difference on the supply side of such an immunological equation could in part be related to the efficiency by which marginal pools of immune cells throughout the body are recruited into the circulation. However, on the demand side of the skeletal muscle, the "pull effect" might rather be modulated by the extent of local inflammation induced, which would be considerably higher in individuals not used to strenuous physical activity[215], a pattern visible in the number of significantly enriched GO-terms at the timepoint immediately following END (Fig. 21A-C). While general terms such as leukocyte migration might engulf receptiveness to all circulating immune cell populations measured in FACS, skeletal muscle seems to be specifically primed for the migration of the myeloid cells of the innate immune system. However, as previously mentioned it has to be kept in mind that in addition to infiltrating immune cells, local resident immune cells of the innate immune system could also be responsible for the discovered effects on transcriptomic level in skeletal muscle.

In summary, we showed that the timing of the increase of immune cell proportion differs between different groups of immune cell populations. Macrophages were part of the group of immune cells

that responded immediately, in control subjects in response to acute resistance exercise, in endurance athletes in response to acute endurance exercise and in strength athletes in response to both. The group-specific response pattern can be partially explained by their above discussed significance to the early, innate immune system-based inflammatory response and the role of and mobilization from marginal pools all over the body that might be optimized in athletes. Furthermore, $CD56^{+dim}$ NK-cells and CD8+ T-cells respond early, in a similar biphasic manner to both END and RES, but in response to END with a larger amplitude which could reflect a certain level of whole-body exercise intensity required to mobilize such cells.

Other cell populations such as CD4+ T-cells peak at the 1h timepoint in response to END and RES but are more endurance specific in the endurance and control groups, while in strength athletes RES can results in a significant increase at the 1h timepoint. It could be speculated that such a difference is due to higher levels of exercise intensity that can be reached in RES by experienced strength athletes only. Furthermore, we show that skeletal muscle, in its role as an endocrine organ as well as recipient of endocrine signalling undergoes a change in gene expression to enable increased mobility of immune cells. However, if this gene expression originates from already infiltrated or resident immune cells or from within skeletal muscle tissue itself remains unclear. Given the extensive immediate increase in circulating immune cells presented here it would not be farfetched to assume that such an effect at least partially originates from an increased supply from the circulation.

4.4 GENERAL DISCUSSION

The papers in this thesis demonstrate the extensive influence that life-long endurance and resistance training in humans has on skeletal muscle transcriptomic, metabolomic and regulomic levels as well as on the immune system. **Papers I-IV** are all based on analysis of skeletal muscle tissue and **paper V** on blood additionally. Naturally, the human body is highly complex - tissues and organs mutually influence each other, and molecular processes mutually influence processes on other molecular levels - locally and systemically. Such complex nature of the human body in conjunction with herein presented results as well as previous and recent literature reinforce the notion of a highly complex network of crosstalk between organs, tissues and compartments of the body. As one example of such systemic connection from a practical sports application, the method of ischemic preconditioning (IPC) has increasingly been used to enhance *systemic* athletic performance. IPC involves controlled brief episodes of *local* ischemia and reperfusion[216], leading to improvements in muscle oxygenation, vasculature and blood flow delivery to active tissues and organs[217]. A recent extensive systematic review of IPC[218], showing extensive benefits of such a local method for systemic performance, provides some performance physiological evidence of widespread tissue and molecular level crosstalk in the context of exercise. Another example of a *systemic* influence of *local* metabolic perturbation is the heat shock proteins (HSPs). HSPs are increased in expression amongst others during homeostatic imbalance following physiological stress such as exercise. They can be found in various organs and tissues locally but also in plasma and mononucleated cells and act as molecular chaperones that have also been described to be involved in cell signaling and regulation of metabolism and thus can mediate a *local* stimulus to a *systemic* effect[219,220].

Previously, many publications approached exercise physiological research questions with a rather isolated focus on individual omics levels of molecular interaction or single tissues only. Recent and ongoing efforts in the field of exercise physiology increasingly acknowledge the idea of a complex biological network and to this end a large NIH-funded consortium, the Molecular Transducers of Physical Activity in Humans (MoTrPAC) was designed to systematically study the underlying molecular mechanisms of physical activity in humans[6]. A recently published study demonstrated the great potential of such a multi-omics approach by allowing novel insights into the orchestrated molecular choreography in response to acute exercise in blood[120]. Considering the future research road map of the MoTrPAC consortium and other research initiatives, extensive new knowledge can be expected from prospective results from skeletal muscle and adipose tissue from individuals across ages and training status and their integration into a more systemic, multi-level view of the exercising human body[221]. The possibility to integrate different tissues and several molecular levels provides extensive possibilities of a more systems biology approach in an attempt to further our understanding of the molecular processes of exercise. Such a systemic approach, like a meta-level analysis of individual tissue responses, could potentially settle disputed concepts such as that of an exercise memory, which so far has largely been approached fairly tissue- or mechanism-centric. As mentioned before, several molecular mechanisms have been individually proposed to be responsible for a potential memory effect, such as CNS learning capacity[8], myonuclear addition[9], epigenetic modifications[7,10] or modified phosphorylase activity as suggested in **paper II**. However, mechanisms, functionality or diseases are often multi-causal rather than monocausal in biological systems[222]. A number of monocausal diseases such as McArdle' disease, a monogenetic disorder impairing the muscles' ability to break down glycogen, or cystic fibrosis do exist. However, more common and sometimes more complex diseases like type 2 diabetes or the metabolic syndrome largely depend on several regulatory networks and systems, spanning different tissues and organs. Similarly, athletic performance depends on a concert of molecular level interactions, covering a large number of tissues and organs. Such a biological principle of complexity could also be applied in an attempt to approach the concept of an exercise memory. Rather than focusing on one specific mechanism, the idea of a memory could potentially be explained better with a more systemic concept in mind, a concept of a "functional systemic memory of exercise". One potential element of such a systemic memory was mentioned already in the 1980s with the concept of the "athlete's gland" – the adaptation of the sympathoadrenal system to regular training and a change of the relationship between sympathetic nervous activity and epinephrine secretion[223]. An adaptation of the endocrine system to long-term high-level training would have great influence on the whole body and its susceptibility to exercise, and therefore the capability to further adapt to exercise with for example increased muscle mass or endurance. Other examples of a potential memory effect are the mechanisms of IPC and heat shock proteins as mentioned above. Similarly, it has previously been shown that long-term training influences enzymatic systems, particularly efficiency of glycolysis, glycogenolysis and gluconeogenesis – metabolic pathways of high importance in physical performance[224]. Any such long-term adaptation that influences other tissues and molecular mechanisms could be seen as contributing to such a functional systemic memory of exercise.

Based on the previously mentioned specific examples and general biological principles, it might be the case that for example adaptations in the sympathoadrenal or metabolic systems could result in an improved adaptational response to resistance exercise, improving hypertrophic processes by optimized local supply of substrates and amino acids and increased adrenal activity[225]. Generally, adaptation to any kind of physical training is largely dependent on protein synthesis rate and such adaptation of different systems and tissues relevant in the context of exercise would follow a long-term choreography rather than happen all at the same timepoint[224,226]. Capillarization of muscle tissue would be increased rather fast, followed by the adaptation of mitochondrial function. A structural adaptation in the form of increased muscle mass could over time be accompanied by the previously proposed concept of myonuclear addition[9,62], adding increased resilience to the muscle mass accumulated. Increased muscle mass amongst others subsequently results in increased total potential for glycogen storage, which could extend local muscle endurance by increased local energy supply. Additionally, but in a delayed manner, tendons and bones would be structurally reinforced to enable the optimized biomechanical utilization of such increased strength. Such a chain of adaptations, happening at system- and tissue-specific speed, would create a certain “drag-effect” of adaptation. As mentioned above, such adaptations have the potential to reinforce each other through their positive systemic effect on exercise performance. However, upon detraining, like loss of muscle mass or demineralization of bones which is delayed in its onset by several months and years, previously acquired structural, cellular and molecular adaptations would be lost in a system-specific time course rather than together at the same timepoint[227]. Immediate de-training effects would largely depend on protein degradation rate, a process that itself could be influenced by long-term training status, and affect glycolytic and mitochondrial enzymes, and later capillary density followed by de-adaptation of hypertrophy. Such a system-specific “drag-effect” with detraining could provide a certain degree of exercise memory which in its duration would depend on the extent of the previously acquired adaptation, giving life-long training athletes larger resilience to such periods of no training in a concept of a functional systemic memory of exercise. However, such a concept would arguably depend on the specific definition of “memory”.

5 CONCLUSIONS

The main findings of this thesis were:

- There is no cumulative effect on STARS gene expression in response to long-term endurance training. Furthermore, there is no difference between men and women in STARS gene and protein expression. (**paper I**)

- A history of unilateral resistance training influences the expression of key regulatory genes, in particular SETD3, MYOD1 and MYOG and novel exercise gene SPRYD7, and increases phosphorylation levels of proteins AMPK and 4E-BP1, involved in protein synthesis. (**paper II**)

- Life-long high-level endurance training results in as much as 1711 and 1097 differentially regulated genes (DEGs) in women and men respectively, compared to untrained controls at rest. Life-long high-level resistance training only results in 26 differentially regulated genes in men compared to untrained controls at rest. (**paper III**)
- Transcriptomic differences between untrained men and women are larger (452 DEGs) than between endurance-trained men and women (135 DEGs). (**paper III**)
- Life-long trained endurance athletes differ from untrained controls largely by increased metabolic activity in the TCA cycle and fatty acid oxidation in both men and women. (**paper III**)
- Short-term training can partially reverse metabolic dysregulation by reversing the direction of gene expression. (**paper III**)

- A life-long high-level exercise background largely influences differentially regulated genes in response to acute endurance or resistance exercise, and athletes have a larger number of DEGs following a type of exercise that they are not normally performing. (**paper IV**)
- Endurance athletes respond to acute endurance exercise with an extensive decrease of metabolic activity directly following acute exercise while untrained controls and strength trained athletes respond with increased metabolic activity at the same timepoint. (**paper IV**)
- HIF1A and MYFfamily transcription factor motifs are specifically responsive to acute endurance and resistance exercise respectively. In endurance athletes, HIF1A activity in response to acute endurance exercise is more extensive and MYFfamily activitiy in response to acute resistance exercise is less extensive compared to strength athletes and untrained controls. Both motifs are furthermore closely associated to distinct sets of interconnected genes. (**paper IV**)

- The response of the immune system to acute exercsise depends to some extent on the type of acute exercise performed and on the training background. Endurance athletes generally respond with stronger changes in immune cell populations in response to acute endurance training. (**paper V**)
- Skeletal muscle as a potential target of immune cell infiltration increases the expression of genes associated with immune cell mobility immediately following acute exercise, especially following endurance exercise. The increased mobility is more extensive in untrained controls compared to life-long trained athletes. (**paper V**)

6 FUTURE PERSPECTIVES AND LIMITATIONS

Apart from this thesis' contribution to the knowledge about exercise-related molecular mechanisms, its inherent limitations highlight a vast potential for future investigations of exercise-associated molecular mechanisms and the benefits of such an improvement of our understanding of exercise physiology and its implication for performance and health.

Understanding molecular processes that translate mechanical signals such as muscle contractions into molecular processes could hold future potentials for strategies or pharmaceutical solutions for people who, for medical reasons, are not able to benefit from the positive impact of exercise due to movement inabilities. While **paper I** of this thesis focused on one specific proposed molecular process of mechano-transduction, the STARS pathway, further investigation into the influence of more intensive resistance exercise protocols could prove to be useful in its understanding. **Paper I** certainly was limited by its statistical power, but several other aspects, for example the direct influence of RhoA on actin polymerization could have additional potential in molecular exercise signaling. Furthermore, biopsy timepoints beyond 24 hours post exercise might provide additional insights into STARS protein dynamics in muscle.

While we showed indications for a possible memory effect of exercise, the underlying molecular mechanisms are still not entirely clear. However, our results show that proteins of the protein synthesis machinery might be differentially phosphorylated and therefore influenced in their activity with previous training. Such an influence could have large implications for the recovery from periods of detraining but has not been investigated so far. To get an idea about the extent of a potential advantage of protein synthesis following acute exercise, more than only one post-timepoint – as used in **paper II** - could be beneficial.

Investigating life-long effects of high-level training is always associated with large logistical and financial challenges, which explains why such investigations are rare. To get around these limitations, rather than a decade-long longitudinal study, in respect to life-long training we chose a cross-sectional study design in **paper III**. However, this could be seen as a limitation since it depended on the reported history of exercise from each subject, nevertheless while also being evaluated by objective performance tests. The design used does not entirely exclude other variables not related to exercise itself, such as genetic variation, health or current performance status. The current performance status, which can be influenced by circadian or seasonal effects could be especially influential in the acute intervention study in life-long trained athletes. Since we were unable to recruit exclusively strength trained women, our **paper III** lacks a group of strength trained women. The difficulty of recruiting strength trained women also influenced the design of the acute intervention study, **papers IV-V**. Because of our difficulties recruiting exclusively strength trained women, papers IV-V were based on male subjects only, as the cross-over design requires a complete endurance and strength group. However, such a shortcoming opens up the potential for a future study to be performed in women only, ideally using a design as similar as possible to enable a high level of comparability.

Such a comparison could have extensive potential for the investigation of sex-based differences in life-long adaptation and acute exercise responses.

As discussed in chapter 4.4, while exercise physiology largely focuses on skeletal muscle, the human body should be seen as a functional unit of interdependencies of tissues and different layers of omics rather than with an isolated view on individual tissues or molecular levels. While we attempted to investigate omics levels other than transcriptomics by using advanced bioinformatic methods, additional investigations on global proteomic, epigenetic, metabolomic or even genomic level could be of great value and would further enable the *ex silico* confirmation of used bioinformatic methods.

Additionally, considering the aforementioned mutual influence of different tissues and organs on physical performance and health, a thorough investigation of for example adipose tissue or blood-borne cytokines and extracellular vesicles could shed new light on the complex crosstalk in the context of physical activity. Especially investigations of the blood as one potentially major mediator of crosstalk could comprise large possibilities for scientific advancement. While we did attempt to investigate such potential communication mechanisms that could be mediated for example by the immune system via circulating immune cells, there is a potential in a more thorough characterization of immune cells and the integration of such results with for example circulating cytokines.

7 POPULAR SCIENCE SUMMARY

The human body is composed of numerous organs, tissues and individual cells in which a multitude of biochemical processes take place. Their coordinated interaction is of great importance to the smooth function of the biological machine that is the human body. The body is made for movement, and the smoothness of its functions and molecular processes can be substantially improved by physical activity. The fundamental functioning, the blueprints of this machine are written in the genetic information, DNA. Even though the sequence of the 3 billion bases that comprise DNA is very similar in all humans, there are small differences between every individual. Noteworthy, the genetic blueprint is not only used to make all of us develop to become humans from the fertilization of the egg, but it is also used on a daily basis as a template for adaptation to environmental alterations, including nutritional or physical activity changes. Such adaptations include the production of proteins that change the structure and function of the body. By such processes, daily habits can over time shape the body in a positive or negative way.

7.1 EXERCISE AND THE BIOLOGICAL MACHINE HUMAN

Regular, sports-specific and progressive training can result in outstanding athletic performance. Additionally, physical activity can over short- or long-term have numerous health benefits, amongst others a more robust cardiocirculatory system, increased bone density, improved immune system function or improved control over metabolic processes and hormonal balance. Some adaptations to regular exercise are more obvious to the eye, like increased muscle mass in resistance-based sports. Others may seem more subtle and reveal themselves rather on a biochemical level, as for example adaptations to endurance exercise. Because of its active role in exercise, skeletal muscle is a classical target of exercise physiological research. In healthy humans, 40 to over 55% of the body can consist of skeletal muscle, a tissue that is highly plastic. Despite the outstanding importance of skeletal muscle, it is important to remember the substantial contribution of other organs and tissue to physical performance capacity, such as adipose tissue, blood as a transport conduit for oxygen and nutrients and immune system organs or metabolic organs such as liver or kidneys. Skeletal muscle consists of a number of structures other than the contractile units, such as capillaries – small blood vessels, local immediately useable energy storages in the form of carbohydrates and lipid droplets and nerves to control the muscle and give feedback to the brain. Nevertheless, the direct response to acute exercise and its high plasticity over a longer period of regular exercise as well as its relatively easy accessibility close to the surface of the body are some of the big advantages of skeletal muscle as a target of research. To do so, small amounts of muscle, some hundred milligrams, can be taken using a special biopsy needle. To study the influence of long-term physical activity it can be especially interesting to investigate highly specialized athletes and to compare those with untrained individuals. Therefore, for the studies on which this thesis is based, muscle and blood samples were taken before and after a session of acute endurance or resistance exercise from the side portion of the knee extensor muscle from very resistance and endurance trained athletes and untrained individuals, to analyze the changes in molecular processes in response to acute activity.

7.2 THE MOLECULAR EFFECT OF LONG-TERM TRAINING

Our results show that long-term endurance training strongly influences how genetic information, genes, are used even before any acute exercise. We revealed that out of about 20 000 genes, approximately 1000 are differently used in endurance trained athletes compared to resistance trained athletes or untrained individuals. Interestingly, only about 50 genes were different when comparing resistance athletes to untrained individuals. These results imply that long-term endurance training but not resistance training largely affects the way we use our genes. It is possible that the main result of strength training is more of structural nature, such as the addition of muscle mass rather than on the molecular level of gene usage. The difference between these groups of people stayed roughly the same at different timepoints following the acute exercise. A closer analysis of the mentioned differently used genes revealed that they are mainly responsible for energy producing processes. The pattern of differences between groups continued into the metabolic level, especially the citrate cycle, a central process in the body's biochemical mechanism to obtain energy from carbohydrates and fatty acids, was affected. Analysis of circulating immune cells in the blood samples showed that athletes reacted slightly different to acute exercise regarding the number of cells circulating in blood, but that the specific kind of acute exercise can also influence this. The number and activation state of the immune cells greatly influences the shape of the immune response following acute exercise which has a large influence on the regeneration of the body. It seems like the immune system of endurance athletes can be activated better by endurance exercise, while in resistance athletes both kinds of acute exercise equally resulted in a mobilization of the immune system.

Furthermore, our results show that already 6-12 months of regular training can result in a large change of how genes are used in people with type 2 diabetes or the metabolic syndrome, which over a longer exercise period can result in a reduction of symptoms and disease severity in these patients. Additionally, our results show that long-term training in some way can build up a kind of "memory effect", which results in an increased speed of improvement of performance after a longer period of inactivity.

7.3 WHAT DOES THIS MEAN PRACTICALLY?

The human body is made for movement, and for the development and maintenance of health and functionality it is important to be and to stay physically active in one form or another. In that context, consistency plays a role, because molecular processes that take place at every moment of our life constantly contribute to laying a foundation on which structure and functionality of our bodies depend. Nevertheless, it seems as if even an exercise novice does get a beneficial effect out of physical activity in the case of for example metabolic diseases such as type 2 diabetes or the metabolic syndrome, which influences symptoms and disease course and has great influence on quality of life. In summary, physical activity can be recommended in almost all situations and for all people, if physically able to. As a general rule, the earlier and the more consistent the better it is.

8 POPULÄRWISSENSCHAFTLICHE ZUSAMMENFASSUNG

Der menschliche Körper setzt sich aus zahllosen Organen, Geweben und einzelnen Zellen zusammen in denen eine Vielzahl von biochemischen Prozessen abläuft deren koordiniertes Zusammenspiel große Bedeutung für die reibungslose Funktion der biologischen Maschine Mensch hat. Diese biologische Maschine auf Bewegung ausgelegt, und wie in einer gut gewarteten Maschine kann die Reibungslosigkeit ihrer Funktionen und molekularen Prozesse durch regelmäßige Bewegung stark verbessert werden. Die grundlegende Funktion, die Baupläne dieser Maschine sind in der genetischen Erbinformation, der DNS, die in jedem Menschen etwas anders ist, niedergeschrieben. Wie jedoch diese Bauanleitung tagtäglich vom Körper angewendet wird kann stark beeinflusst werden, zum Beispiel von Umwelteinflüssen, Ernährung und sportlicher Aktivität. Diese verschiedenen Faktoren beeinflussen unter anderem die Produktion von Proteinen, kleine Biomoleküle aus denen der Körper aufgebaut ist, und legen damit jeden Tag einen kleine Teil der Grundlage der Struktur und Funktion des Körpers. Durch diesen Prozess können mit der Zeit tägliche Gewohnheiten den Körper und seine Funktionen formen und positiv oder negativ beeinflussen.

8.1 SPORT UND DIE BIOLOGISCHE MASCHINE MENSCH

Regelmäßiges sportspezifisches und progressives Training kann so zu herausragenden sportlichen Leistungen befähigen. Zusätzlich kann Sport, je nach Sportart kurz- und langfristig zahlreiche gesundheitliche Vorteile haben, unter anderem ein robusteres Herz-Kreislaufsystem, erhöhte Knochendichte, verbesserte Funktion des Immunsystems oder verbesserte Kontrolle über Stoffwechselprozesse und den Hormonhaushalt. Während manche Anpassungen an regelmäßigen Sport äußerlich offensichtlicher sind, zum Beispiel erhöhte Muskelmasse in Kraftsportarten, sind andere subtiler, und werden eher auf molekularer Ebene offenbar, zum Beispiel Anpassungen and Ausdauersportarten die sich stark auf biochemische Prozesse auswirken die dem Körper Energie zur Verfügung stellen. Aufgrund seiner aktiven Rolle in körperlicher Bewegung ist ein klassisches Zielorgan der sportbiologischen Forschung der Muskel. In gesunden Menschen bestehen 40 bis über 55% des Körpers aus Skelettmuskeln, ein Gewebe das seine Anpassungsfähigkeit seiner hohen Plastizität verdankt. Trotz dieser herausragenden Bedeutung des Skelettmuskels sollte die große Bedeutung anderer Organe und Gewebe für sportliche Leistungsfähigkeit und Gesundheit nicht außer Acht gelassen werden, wie zum Beispiel Fettgewebe, das Blut als Transport- aber auch Immunorgan und Stoffwechselorgane wie die Leber und Nieren. Skelettmuskeln selbst wiederum beinhalten verschieden Strukturen, abgesehen von den mikroskopisch kleinen biomechanischen Einheiten, welche die Kontraktion ermöglichen unter anderem aus Kapillaren – kleinen Blutgefäßen, Energiespeicher und Nerven zur Steuerung des Muskels. Die hohe Plastizität, Veränderungen im Muskel als unmittelbare und direkte Folge von sportlicher Betätigung, und die einfache Zugänglichkeit an der Körperoberfläche sind einige der Vorteile von Muskelgewebe als Forschungsobjekt. Kleine Mengen Muskel, einige hundert Milligramm werden dazu mit Hilfe einer speziellen Biopsienadel aus dem Körper entnommen. Um den Einfluss von langfristiger physischer Aktivität auf die molekularen Prozesse zu verstehen kann es besonders interessant sein, hochspezialisierte Athleten zu untersuchen und mit untrainier-

ten aber gesunden Versuchspersonen zu vergleichen. In mehreren Studien dieser Doktorarbeit wurden daher Muskelbiopsien vor und nach einer Einheit Ausdauer- oder Krafttraining aus dem seitlichen Teil der Kniestreckermuskulatur von besonders ausdauertrainierten Athleten, besonders kraftsporttrainierten Athleten und untrainierten Versuchspersonen entnommen, zusammen mit Blutproben diese Veränderungen der molekularen Prozesse durch diese Aktivität analysieren zu können.

8.2 DER MOLEKULARE EFFEKT LANGFRISTIGEN TRAININGS

Unsere Ergebnisse zeigen, dass langfristiges Ausdauertraining stark beeinflusst wie genetische Erbinformation, Gene, verwendet wird – schon bevor eine Trainingseinheit absolviert wird. Von den ungefähr 20-25000 menschlichen Genen zeigte sich, dass ungefähr 1000 statistisch mehr oder weniger verwendet wurden verglichen mit Kraftsportathleten oder Untrainierten. Interessanterweise gab es nur in ungefähr 50 Genen einen Unterschied zwischen Kraftsportathleten und Untrainierten. An verschiedenen Zeitpunkten nach einer Trainingseinheit blieb dieser Unterschied blieb ungefähr gleich. In einer genaueren Analyse dieser Gene stellte sich heraus, dass diese Gene hauptsächlich für Energiebereitstellungs- und -produktionsprozesse verantwortlich sind. Dieses Muster setzte sich auf Stoffwechselniveau fort, besonders betroffen war der Citratzyklus, ein zentraler Prozess in der körperlichen Energiebereitstellung aus Kohlenhydraten und Fett. In dem entnommenen Blut wurden Immunzellen analysiert und es zeigte sich, dass Athleten, ob kraft- oder ausdauertrainiert, leicht anders auf eine Trainingseinheit reagieren bezüglich der Art und Menge von Immunzellen die mobilisiert werden und im Blut zirkulieren, aber auch die Art der Trainingseinheit einen gewissen Einfluss hat. Die Anzahl und der Zustand dieser Zellen kann die Immunantwort nach einer Trainingseinheit stark beeinflussen, was einen großen Effekt auf die Regeneration des Körpers haben kann. Es scheint, als ob sich das Immunsystem von Ausdauerathleten besser mit Ausdauersport aktivieren lässt, während für Kraftsportathleten beide Arten von Sport gleichermaßen zur Mobilisation führte.

Darüber hinaus zeigen unsere Ergebnisse, dass bereits 6-12 Monate regelmäßiges Training bei Patienten mit Diabetes Typ II oder dem Metabolischen Syndrom eine starke Veränderung in der Art wie Gene verwendet werden verursachen kann, was langfristig dazu führen kann Symptome und Schwere dieser Krankheiten zu lindern und die Stoffwechselgesundheit dieser Patienten zu verbessern. Weiters zeigen unsere Ergebnisse, dass langfristiges Training im zunehmenden Maße zu einer Art „Erinnerungseffekt“ führen kann, der dazu führt dass sich der Körper zu einem gewissen Maß auf frühere sportliche Aktivität aufbauen kann, was selbst nach längeren Zeiträumen von Inaktivität ermöglichen kann frühere Leistungsniveaus schneller zu erreichen bzw. Leistung zu verbessern.

8.3 WAS BEDEUTET DAS PRAKTISCH?

Der menschliche Körper ist für Bewegung gemacht, und für die Entwicklung und Erhaltung von Gesundheit und Funktionalität ist es wichtig regelmäßig in der einen oder andern Form physisch Aktiv zu sein und zu bleiben. Dabei ist Kontinuität ein Faktor, denn molekulare Prozesse die in jedem Moment unseres Lebens ablaufen tragen permanent zum Aufbau des Fundaments bei auf das sich die Struktur und Funktionalität unseres Körpers stützt. Jedoch zeigt sich, dass selbst die Neuaufnahme körperliche Aktivität im Fall von metabolischen Krankheiten, wie zum Beispiel Diabetes Typ II oder

dem Metabolischen Syndrom starke positive Effekte auf Krankheitsverlauf und Symptomatik und damit auf die Lebensqualität haben kann. Im Grunde kann Sport in so gut wie jeder Situation und für alle Menschen, soweit möglich, empfohlen werden und generell gilt je frühzeitiger und kontinuierlicher desto besser. Diese Vorteile schlagen sich nieder auf viele verschiedene Organe, Gewebe, molekulare Effekte und können zu Gesundheit und verlängerter Lebensqualität beitragen.

9 ACKNOWLEDGEMENTS

I would like to thank all the **volunteers** participating in the studies for our five papers for their bravery, their time and effort in enduring our demanding experimental designs, their sweat and for literally giving a piece of themselves to science. This research would not have been possible without you, thank you very much!

I would also like to thank everybody who in any way contributed to this thesis and my research,

especially my great *main supervisor* **Carl Johan Sundberg** - thank you for giving me the opportunity to work in such an exciting field of research with such great scientific challenges touching on so many different other disciplines of biomedicine! It is a pleasure to work in such a great and inspiring environment and to be able to connect passion for exercise with excitement for science. Thanks for your thought-challenging inspiration and invaluable support during the last five years!

my fantastic co-supervisor **Jessica Norrbom**, who is always there for me for questions and discussions about science and teaching. The way you handle so many things at the same time is impressive! Thank you for your extensive knowledge about exercise science and all the good atmosphere you bring to the lab!

who both have guided me through these years of scientific and personal development.

and my co-supervisor **Jan Nedergaard**, who in our meetings always encouraged critical thinking and helped a lot in my understanding of tissue biology.

My *co-author*s and colleagues across the world – our former and current group members. **Eva-karin Gidlund**, thanks for your shining personality that just made every day even better and your contagious passion for science. And for taking me along for my great first conference in Boston. **Mark Chapman**, who came to our lab the same time as me and shared that "coming to Sweden" experience, thanks for your great work in paving the path and laying the foundation for the great projects we have been and are running, and for the many dinner parties. Gut Mark! **Eric Emanuelsson**, our newest group member, thanks for holding out together with me and your reliability in over a year of early-morning biopsies and exercise interventions at the hospital. The lab would be pretty empty without you. Also, my plants would be dead. **Malene Lindholm,** it is great to see what you have achieved here and at all your other stations after KI! Thanks also to our former group members **Sara Mijwel** and **Mia Ydfors** and to my colleague in the spirit of organization **Charlotte Schönbeck**, our lab manager for many years.

Erik Arner, thank you for welcoming me into your group and sharing your knowledge and experience in the power of bioinformatics. The course I took at your lab first got me hooked to R, but the research visit really put things on a different level. Thanks for the amazing opportunity to live in Japan and hopefully to more awesome collaborations in the future and a good paper to come out of the

previous one! Also, many thanks to **Bogumil Kaczkowski** and **Andrew Kwon**, for showing me the way and taking the time helping me solve my programming problems, I'll stick to pipes over loops!

Many thanks also to all the people at Huddinge, thanks **Mats Lilja** for showing me around the exercise lab and helping me keep up the logistical end of my studies and **Thomas Gustafsson** for providing us with such a great platform for our exercise interventions and sample collections. **Helene Rundqvist,** thanks for your great input in immunology and for giving me my first lab project here at KI (that actually involved shooting a laser at tissue samples, how fancy is that!) and **Laura Barbieri** for showing me how to do FACS. **Muhammad Arif**, it was great to work together with you on the analysis of our complex study design, thank you for your great support! And **Niklas Psilander**, thank you for your interesting studies and ideas and for good collaboration over the years! Also thanks to my other colleagues at our department and KI, great experience from many hours of teaching with **Iréne Lund**, our great and always helpful PhD student administrator **Sofia Petterson**, and **Niklas Ivarsson** for his help with these tricky biochemical assays, sad you are not here anymore! Also, all the **core facilities** and their staff at KI and SciLife, what great possibilities we have through you!

Thanks to my dedicated students over the years, **Seraphina, Leo** and **Elia** for your contribution to our labs work and for the experience of being in the supervisor role myself.

Getting to this point means going through many stations that are part of the journey some of which by chance put me on the path to exercise physiology, starting with some great teachers at high school that sparked my curiosity in science. Thanks to my first supervisors **Armin** and **Dirk**, who guided me through my bachelor thesis and my first contact with laboratory science and all the people I got to know while studying at the University of Innsbruck, especially **Mikel** and **Alex**. Also, thanks to **Clemens**, who I spent a lot of time working out with while I got more and more excited about how great exercise is. **Ricardo**, thanks for sparking the idea of connecting my interests in exercise and science by telling me about the research at the Sports University in Cologne. Thanks to the people that I got to know there, my master thesis supervisors and group members **Jonas, Patrick** and **Carmen** and the people I got to know during my time in Cologne, **Jan**, **Jens**, **Adrian** and everybody else. For being a truly great friend for so many years, someone I could always talk to, rely on and for everything else, especially thank you **Markus**! I'm also very thankful for the people I got to know for already such a long time in Stockholm that, apart from my colleagues at KI made feeling at home in a new country so much easier, **Isak, Mehdi, Ibbe** and all the other great people I met in the past five years.

And of course, my family – **Mama**, **Papa**, thanks for supporting me over all these years, my brother **Tobias** and my sister **Judith** and my (so far) three nephews, the next generation that we do all this science for. My aunt and – can I say that now? – fellow scientist **Barbara** who I'm sure will take a closer look at some of my papers, and my **grandparents**, who would have been really happy and proud to see this book.

Finally, thanks for all the financial support and enabling the work in this thesis to **Karolinska Institutet**, the **Swedish Center for Sports Research**, the **Swedish Research Council** and the **Swedish Society of Medicine**.

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